Biomarker, Metabolomics and Doping
A Novel Approach to Detect Drug Misuse

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Biomarker, Metabolomics and Doping: A Novel Approach to Detect Drug Misuse

By

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Abstract

Traditional anti-doping assays target known banned drugs and endogenous molecules. A discovery platform such as metabolomics can be employed to find small molecule biomarkers that are related to the function of drugs of misuse. Biomarkers can be endogenous surrogate metabolites which have the potential to facilitate the discovery of new doping agents with biological activities similar to related named prohibited substances, as well as improving the sensitivity and prolonging the detection time window. Working towards this end, the focus of this thesis was to apply metabolomics to the anti-doping field in order to enhance detection of drug misuse in sport, in particular focusing in methodology that would help discover endogenous biomarkers, namely: sample information, quality, storage, transport, and multivariate/univariate statistics to mine significant biomarkers from metabolic data.

The first part of the thesis explored analytical method-development in metabolomics, overall assessing the amount and quality of the metabolic information acquired in small sample volumes using paper spots. Methods for high resolution and high mass accuracy instrument were also studied to aid with the identification of molecules. After this, paper spots were also assessed for storage stability and compared with the conventional sampling method. From these analyses it was concluded that paper spots are a good option when limited sample is available, due to reproducibility, storage and transport advantages, however there is a loss of metabolic information when compared to liquid samples. The high mass

accuracy did help with identification although the method was not tested in fragmentation mode.

Then the application of multivariate statistics to mine biomarkers from "doping fingerprints" was studied in detail. Firstly, a small clinical trial with longitudinal samples containing one (endogenous and exogenous) metabolite in urine, GHB, was "fingerprinted". The aim was to find biomarkers that could be identified for a longer detection window than GHB in urine. A battery of multivariate tools was used to mine the data. Several metabolic features, albeit partly unidentified, showed interesting results, however it was concluded that a new study design with diet control and a placebo group, was needed to validate these features.

Lastly, samples from athletes were analysed and were grouped as salbutamol detectable and non-detectable specimens. Since this study was based upon the shortcomings of the previous GHB study, a strategy to diminish false positive biomarker discovery was included by adding a salbutamol spiked group in the experimental design. Results revealed a potential endogenous biomarker of salbutamol, hypoxanthine, with a ROC of 79 % prediction. These promising results need further validation with a subsequent clinical trial.

Overall these preliminary studies show the potential of metabolic fingerprinting in anti-doping. The findings have shown that metabolomics is a valuable tool in the discovery of surrogate biomarkers in doping.

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List of Abbreviations

2D-DIGE	Two-dimensional fluorescence difference gel electrophoresis
2-DE	Two-dimensional electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
5αAdiol	5α-androstane-3α,17β-diol
5βAdiol	5β-androstane-3α,17β-diol
A	Androsterone
AAF	Adverse analytical findings
AAS	Anabolic androgenic steroids
ABP	Athlete Biological Passport
ABPS	Abnormal Blood Profile Score
ABT	Autologous blood transfusion
ADRV	Anti-Doping Rule Violations
ATF	Atypical findings
ATP	Adenosine triphosphate
BMI	Body mass index
BPI	Base Peak Intensity
BPI	Base peak intensity
CAS	Court of Arbitration for Sport
CE-MS	Capillary electrophoresis–mass spectrometry
CERA	Continuous erythropoiesis receptor activator
CSF	Cerebrospinal fluid
DBS	Dried biofluid spots
DEHP	Di-(2-ethyhexyl) phthalate
DESI	Desorption electrospray ionization
DESI	Desorption electrospray ionization
DET	Salbutamol detectable samples
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
E	Epitestosterone
EAAS	Endogenous anabolic androgenic steroids

ELISA	Enzyme-linked immunosorbent assay
ESAs	Erythropoiesis-stimulating agents
ESI	Electrospray ionization
Etio	Etiocholanolone
F/T	Freeze-thaw
FPR	False positive rate
FT(-ICR)-MS	Fourier transform (ion cyclotron resonance) mass spectrometers
FWHM	Full width at half maximum
GBL	Gamma-butyrolactone
GC	Gas chromatography
GH	Growth hormone
GHB	Gamma-hydroxybutyric acid
GHRP	Growth hormone–releasing hormone
HBT	Homologous blood transfusion
HCT	Haematocrit
HGB	Haemoglobin
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
HRAM	High-resolution accurate-mass
HRMS	High resolution mass spectrometry
Hx	Hypoxanthine
IA	Iimmunoassy
ICTP	Collagen-I telopeptides
IEF	Isoelectric focusing
IGFB3	IGF-binding protein3
IGFs	Insulin-like growth factors
IRF	Immature reticulocyte fraction
IS	Internal standard
IVDE	In-vial dual extraction
LC-MS(/MS)	Liquid chromatography – (tandem) mass spectrometry
m/z	Mass-to-Charge Ratio
MAIIA	Membrane-assisted isoform immunoassay
MALDI	Matrix-assisted laser desorption/ionization

MALDI	Matrix-assisted laser desorption/ionization
MANOVA	Multivariate analysis of variance
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
miRNAs	microRNAs
mRNA	messenger RNA
MS	Mass spectrometry
MSI	MS imaging
MSI	Mass spectrometry imaging
NGS	Next generation sequencing
NIMS	Nanostructure-initiator mass spectrometry
NIMS	Nanostructure-initiator mass spectrometry.
NMR	Nuclear magnetic resonance
NON-DET	Salbutamol non-detectable samples
OFFS	OFF-hr Score
OPLS-DA	Orthogonal partial least-squares – discriminant analysis
PCA	Principal component analysis
PERMANOVA	Permutational multivariate analysis of variance
P-III-NP	Procollagen type III amino-terminal propeptide
PLS-DA	Partial least squares discriminant analysis
QC	Quality control
qRT-PCR	Quantitative real-time polymerase chain reaction
QTOF-MS	Quadrupole Time-of-flight Mass Spectrometry
RBC	Red blood cells count
RDW-SD	Red cell distribution width (standard deviation)
RET	Reticulocytes
RET %	Percentage of reticulocytes
RET#	Reticulocytes count
rhEPO	Recombinant human erythropoietin
rhGH	Recombinant human growth hormone
RMSEE	Root mean square error of evaluation
RMSEP	Root mean square error of prediction
ROC	Receiver Operating Characteristic

RP	Reverse phase
RSD	Relative standard deviation
RT	Retention time
SAGE	Serial analysis of gene expression
SARMS	Selective androgen receptor modulators
SB	Salbutamol
SD	Standard deviation
SDS/SAR-PAGE	Sodium dodecyl sulphate/ sarcosyl polyacrylamide gel electrophoresis
SELDI-TOF	Surface enhanced laser desorption/ionization time-of-flight mass spectrometry
SEM	Standard error of the mean
SFC	Supercritical fluid chromatography
S/N	Signal to noise ratio
SPI	Controls spiked with salbutamol
SRM	Selected reaction monitoring
sTFr	Soluble transferrin receptor concentration
T	Testosterone
TOF-MS	Time-of-flight mass spectrometry
TPR	True positive rate
TUE	Therapeutic Use Exemption
UHPLC	Ultra-high performance liquid chromatography
VIP	Variable importance in the projection
WADA	World Anti-Doping Agency

Publications from this thesis

Metabolic phenotype of the healthy rodent model using in-vial extraction of dried serum, urine, and cerebrospinal fluid spots.

Arundhuti Sen[§], Yaoyao Wang[§], Kin Chiu, Luke Whiley, David Cowan, Raymond Chuen Chung Chang and Cristina Legido-Quigley (**§ co-first author**)

***Analytical Chemistry*. 2013 Aug 6;85(15):7257-63.**

LC-MS based metabolomics discovers purine endogenous associations with low dose salbutamol in urine collected for anti-doping tests.

Yaoyao Wang, Richard Caldwell, David A. Cowan and Cristina Legido-Quigley

***Analytical Chemistry*. 2016 Feb 16;88(4):2243-9.**

Chapter 1 Introduction

1.1 Development and challenges of anti-doping tests in sport

The development of sports has always been accompanied by attempts to use prohibited substances and methods, i.e. doping. According to historians, the idea of doping dates back to the ancient Olympic Games more than 2000 years ago, when some athletes drank potions made from plant extracts, and even ate raw animal testicles, in order to enhance their sports performance (Kelland 2012). Athletes in the modern Olympics era have continued this practice, but with new doping strategies. This may be the result of advances in scientific research, which have led to the discovery and use of performance-enhancing substances.

The International Association of Athletics Federations (IAAF) was the first international sports federation (IF) to formally ban doping in 1928 (Bowers 2012), but it was not until the 1960s that the fight against doping in humans using analytical methods commenced following the amphetamine-related death of a Danish cyclist during the Olympic Games in Rome, and another fatal incident in the Tour de France in 1967 (Beckett 1981). Since then various actions and measures have been initiated to control the misuse of performance-enhancing substances in sport, and a brief history of some remarkable movements is shown in **Table 1-1**.

Table 1-1 Brief history of doping control in human sport

Year	Event
1928	The IAAF becomes the first federation to ban doping
1966	The IAAF, the Union Cycliste Internationale (UCI), and the Fédération Internationale de Football Association (FIFA) introduce urine drug tests in their respective championships
1967	The International Olympic Committee (IOC) institutes its Medical Commission and sets up the first list of prohibited substances
1968	Drug tests introduced at the Olympic Games
1970s	Marked increase in the number of doping-related disqualifications after the introduction by the IOC of anabolic steroids to its list of prohibited substances
1980s	Introduction of out-of-competition testing
1986	Blood transfusion banned by IOC
1990	rhEPO included in the IOC's list of prohibited substances
1990s	Introduction of blood tests
1999	WADA is established succeeding Medical Commission of IOC
2004	The World Anti-Doping Code is adopted worldwide
2005	United Nations Educational, Scientific and Cultural Organization (UNESCO) adopts the International Convention against Doping in Sport
2008	The UCI is the first federation to introduce the haematology module of Athlete Biological Passport (ABP)
2012	Introduction of indirect biomarker test of growth hormone using IGF-1 and P-III-NP
2014	Introduction of steroids module of ABP

IFFA: the International Association of Athletics Federations; rhEPO: recombinant human erythropoietin; WADA: World Anti-doping Agency; IGF-I: insulin-like growth factor-I; P-III-NP: procollagen type III amino-terminal propeptide.
Table adapted from (Sottas, Robinson et al. 2011) with supplements

The techniques and coverage of compounds in anti-doping tests have vastly improved in the past 50 years. Immunological methods and gas chromatography (GC) –based techniques were initially used as preferential screening methods to detect prohibited substances, although they were limited to non-endogenous stimulants and narcotics (Botrè, de la Torre *et al.* 2016). This was followed by the rapid and revolutionary development of gas chromatography-mass spectrometry (GC–MS), which held a dominant position in routine doping controls from the 1980s until almost the end of the 20th century (Thevis, Thomas *et al.* 2011). It overcame some major challenges using sophisticated derivatization strategies, such as analysing heavy volatile or polar compounds.

The list of banned substances has grown significantly during the past two decades, and the incapability of GC–MS to identify high molecular weight, thermolabile, non-volatile analytes and peptide hormones has driven anti-doping scientists to develop techniques based on liquid chromatography–(tandem) mass spectrometry [LC–MS(/MS)]. Moreover, the consideration of time and manpower, as well as the necessity to use hazardous derivatizing reagents in GC, has further reinforced the leading role of LC–MS (/MS) in sport anti-doping testing (Thevis and Schänzer 2007, Hemmersbach 2008).

In today's anti-doping laboratories, rapid and high-throughput technologies are utilised to routinely screen for and quantify hundreds of endogenous and exogenous compounds prohibited by the World Anti-Doping Agency (WADA) **(Table 1-2)**. LC–MS(/MS) and particularly ultra-high performance liquid chromatography (UHPLC–MS(/MS)) have become the instruments of choice in the comprehensive identification of prohibited drugs in doping control specimens,

due to their imperative capabilities compared to the inherent limitations of other commonly-used strategies, such as immunology and GC–MS(/MS) based detection methods (Thevis, Thomas *et al.* 2013).

Table 1-2 World Anti-Doping Agency Prohibited List - January 2016
Information obtained from (WADA 2016)

Substances			Methods
Prohibited at all times	Prohibited in-competition only	Prohibited in particular sports	Prohibited at all times
S0. Non-Approved Substances	S6. Stimulants	P1. Alcohol	M1. Manipulation of Blood and Blood Components
S1. Anabolic Agents	S7. Narcotics	P2. Beta-Blockers	M2. Chemical and Physical Manipulation
S2. Peptide Hormones, Growth Factors and Related Substances	S8. Cannabinoids		M3. Gene Doping
S3.Beta-2 Agonists	S9. Glucocorticoids		
S4. Hormone and Metabolic Modulators			
S5. Diuretics and Other Masking Agents			

Despite the continual improvement in analytical techniques, there is no sign of doping trend coming to an end. As soon as a detection method is proven to be successful, the dopers move on to even more sophisticated compounds and methods. The list of doping substances and methods has continued to grow since the first anti-doping test in the 1960s, and today's anti-doping laboratories tend to focus on using sophisticated analytical methods in an attempt to identify prohibited substances (or methods) or the corresponding metabolites in athletes' samples. However, the ever-changing improvements have always been matched by the

increasingly sophisticated methods or drugs available to cheat due to the advances in science and medicine, which means that the test methods are constantly responding to new threats. This is often described as a race against cheaters who are always one step ahead.

Another major issue facing doping test is the doping using biological therapeutic agents or technologies, including protein therapeutics such as insulins, recombinant human growth hormone (rhGH), insulin-like growth factors (IGFs), recombinant human erythropoietin (rhEPO) and blood doping, which highly resemble their endogenous counterparts, making the detection and proof of doping much more complicated. The potential abuse of gene therapy has also become a serious concern as recently reviewed Brzeziańska *et al.,* pointing out the lack of credible and effective tests to detect gene doping (Brzezianska, Domanska *et al.* 2014).

Facing these new threats, the sports authorities and testing laboratories have increasingly recognised that to counter doping in sports, targeted screening for ever-increasing number of compounds cannot remain the method of choice and, radically different approaches are in demand (Teale, Barton *et al.* 2009).

Anti-doping laboratories in recent years have started modifying their screening approach from targeted data acquisition to total data capture using high resolution mass spectrometry instruments (Virus, Sobolevsky *et al*. 2008, Peters, Oosterink *et al*. 2010, Vonaparti, Lyris *et al*. 2010, Musenga and Cowan 2013, Thevis, Kuuranne *et al*. 2014). This permits total data capture and eliminates the requirement for *a priori* selection of the compounds to be targeted. Though this adds tremendous flexibility to the methods and enables retrospective analysis of

samples, the data processing step still depends on a targeted approach, where "targeted" extracted ion chromatograms are generated from the "untargeted" acquired data. Rather, a new paradigm shift in sports anti-doping tests has come to view, from the direct identification of banned substances in the biofluids of athletes to the detection of abnormalities in biomarkers that potentially indicate doping, which is considered one of the most promising strategies that could aid for the emerging threats, leading to significant coordinate funding by WADA (WADA 2016).

1.2 Biomarker and its discovery approach

1.2.1 Definition of a biomarker and its potential to control doping

A biomarker is defined as "a molecule that indicates an alteration of the physiological state of an individual in relation to health or disease state, drug treatment, toxins, and other challenges of the environment" (Zolg and Langen 2004). However, it can be extended to any measurable parameter that has significantly changed in a living system in response to a challenge. Another definition of biomarker recently by NIH-FDA biomarker working group is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how an individual feels, functions, or survives." (FDA-NIH 2016) The application of biomarkers as surrogate indicators of the a bodily state is a well-developed field, especially in diagnosing diseases such as cancer (Zhang, Li *et al.*

2007) and Alzheimer's (Whiley and Legido-Quigley 2011). In the same way that disease-related biomarkers can assist the diagnosis of disease pathology, the physiological changes caused by doping agents or technologies are also invaluable tools to mirror the misuse by utilising specifically selected biomarkers. Since the effect of the doping agent generally remains detectable in the body in a longer time scale than the substance itself, biomarkers are essential if the drug or method has a very short detection window.

The biomarker method is the first indirect test that has been developed to detect misuse of an endogenous substance. The discrimination principle is not based on a distinction between the exogenous substance and its endogenous counterpart, but rather on the physiological effect induced by the intake of the exogenous substance on some selected biological markers. In fact, this is not a new concept because the sports authorities have used a concentration of testosterone/epitestosterone (T/E) to detect the administration of anabolic steroids since the 1980s, which will be discussed later in section **1.3.1**.

The potential of the biomarker method to indicate the use of a certain category of drugs is also particularly attractive. Today's anti-doping analytical methods need to be continuously modified to address every new agent that is added to the prohibited list, which makes the detection methods more and more complex, as well as time-consuming and expensive. Furthermore, so-called "designer drugs" from underground laboratories could circulate for quite a while among athletes and their trainers before fully coming to the public's attention. However, most of these emerging threats are analogues of known banned substances, with which they share the same functional group, causing similar biological reactions in the body. Thus,

monitoring one/several biomarker(s) that are indicative of a certain biological process caused by the corresponding drug category could potentially be a rapid screening method for targeting athletes with a suspicious profile, followed by an established confirmatory method (Riedmaier, Becker *et al.* 2009).

However, it is somewhat difficult to utilise biomarkers to confirm Anti-Doping Rule Violations (ADRV) because these methods must be proven to be totally reliable in view of all the heterogeneous and confounding factors, with a very high specificity. This is an extremely complex and time-consuming process, which is still difficult to achieve. Nonetheless, some biomarker methods have already been successfully applied to confirm ADRVs, such as the biomarker approach to detect the human growth hormone (hGH) and the Athlete Biological Passport (ABP) to detect autologous blood transfusion (ABT) and gene doping, which will be discussed later in section **1.5**.

1.2.2 Large-scale "omics" approaches to discovery novel biomarkers

The discovery of novel biomarkers in doping mainly entails the use of large-scale "omics" technologies, including genomics, transcriptomics, proteomics and metabolomics (**Figure 1-1**), as directed and supported by WADA. The "omics" approaches rely on high-throughput technologies to simultaneously detect, characterise and quantify thousands of molecules in biosystems. A simplified overview of the "omics" cascade can be found in **Figure 1-1**. In summary, a single gene coding for a protein, for example an enzyme, can be transcribed to mRNA multiple times. In turn, each strand of mRNA can be translated many times into the relevant proteins. Finally, each resultant enzyme molecule could potentially lead to

the catalysis of a large number of metabolic reactions, resulting in the perturbation of small molecule metabolites. At the same time, feedback, crosstalk, and other complex interactions of those stages can occur.

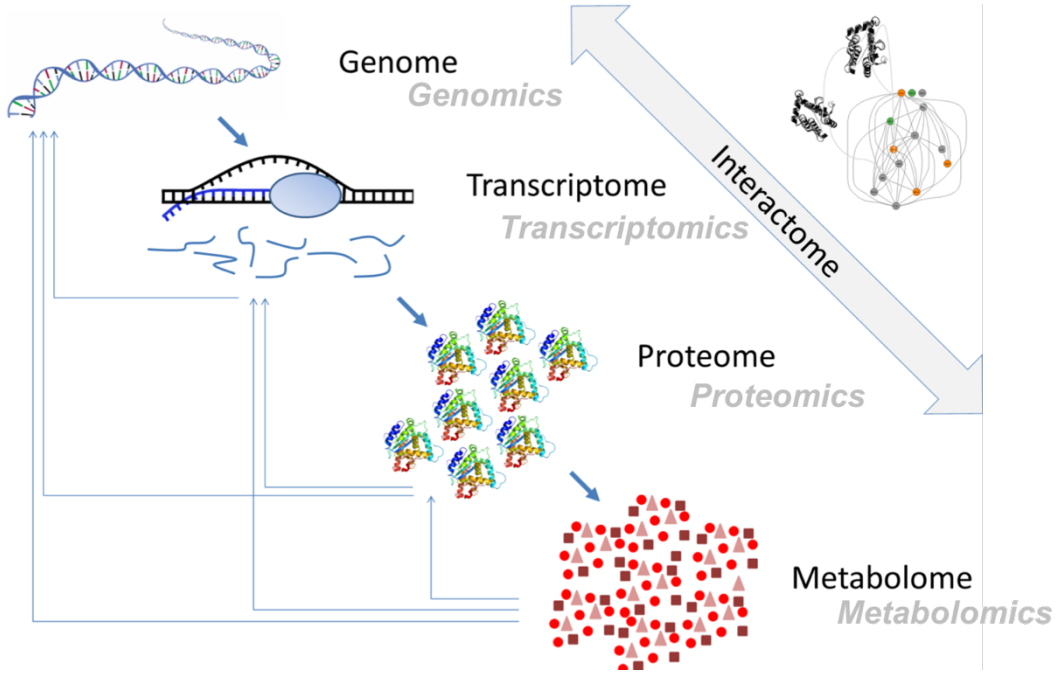

Figure 1-1 A simplified overview of the "omics" cascade. Feedforward, feedback, crosstalk, and other complex interactions of those stages can occur.

The "omics" approaches are widely used in disease diagnostics as well as for monitoring disease progression and therapies. In terms of doping control, since changes in the level of molecules are assumed to be signs of doping, a number of studies have been conducted on the application of an "omics" strategy. C. Reichel summarised research using "omics" strategies before 2011 and demonstrated the potential of "omics"-sciences in the anti-doping field (Reichel 2011).

While the ultimate goal is the biomarker discovery of substancs or methods that are difficult to detect (e.g. hGH and autologous blood transfusion), the longitudinal monitoring of changes in "omics-patterns" can provide a new horizon for anti-

doping control within the frame work of ABP, details of which will be provided in section **1.5.5**.

1.2.2.1 Genomics and transcriptomics

Genomics deals with the exploration of genome, which are organised in genes. The information for the synthesis of proteins carried by genes need to be first transcribed into RNA, especially messenger RNA (mRNA). The entire set of expressed RNA in specific cells, tissues, or the whole organism, is called transcriptome (Reichel 2011). The term "transcriptomics" generally refers to the science dealing with characterization and quantification of transcriptome. Since doping substances have been shown to influence mRNA expression, it has been suggested that these changes can be detected by transcriptional profiling (Friedmann, Rabin *et al.* 2010), using expression profiling technologies such as DNA microarrays, serial analysis of gene expression (SAGE) and next generation sequencing (NGS), followed by targeted quantitation tool of quantitative real-time polymerase chain reaction (qRT-PCR). Genomics/transcriptomics approaches have been largely applied in the anti-doping research, such as the indirect detection of hGH (Thakkar, Butt *et al.* 2008, Mitchell, Nelson *et al.* 2009), rhEPO (Varlet-Marie, Audran *et al.* 2009, Bailly-Chouriberry, Noguier *et al.* 2010), autologous blood transfusion (Pottgiesser, Schumacher *et al.* 2009) and gene doping (Baoutina, Coldham *et al.* 2010, Beiter, Zimmermann *et al.* 2011, Perez, Le Guiner *et al.* 2013). It is worth to mention that recently several groups have suggested that the small, non-coding circulating microRNAs (miRNAs), which enable post-transcription regulation of gene expression by the suppression of specific target

mRNAs, have great potential to be a new generation of anti-doping biomarkers (Leuenberger, Jan *et al.* 2011, Leuenberger, Robinson *et al.* 2013, Kelly, Haverstick *et al.* 2014, Leuenberger and Saugy 2015).

1.2.2.2 Proteomics

The science of proteomics generally deals with the large-scale determination of gene and cellular function directly at the protein level (Aebersold and Mann 2003). The huge potential of protein biomarkers to revolutionize clinical practice and thus improve patient care through molecular based diagnostics has been thoroughly described. Protein profiling provides a powerful method for characterizing the complete set of proteins expressed in a biological system (Kohn, Azad *et al.* 2007), as well as detecting individual isoforms of each protein, thus in anti-doping research, it can improve the specificity and possibly the detection window. The key techniques employed are mainly two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for protein separation followed by MS(/MS) for identification (Aebersold and Mann 2003, Kay and Creaser 2010), other analytical techniques such as two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), high performance liquid chromatography (HPLC) and stable isotopes labelling are also widely used (Reichel 2011). The doping-related proteomics research so far have been largely dedicated to the biomarker discovery for the misuse of recombinant human growth hormone (Ding, List *et al.* 2009, Kay, Barton *et al.* 2009, Baumann 2012). Other topics such as blood transfusion(Salamin, De Angelis *et al.* 2016), erythropoietin (Christensen, Sackmann-Sala *et al.* 2011, Christensen, Ludvigsen *et al.* 2015), anabolic steroids (Imperlini, Mancini *et al.*

2010, McGrath, van Meeuwen *et al.* 2013), proteases (Kohler, Thomas *et al.* 2009), and endurance exercise (Kohler, Walpurgis *et al.* 2010) have also been described.

1.2.2.3 Metabolomics

Metabolomics originated from the idea that changes in tissues and biological fluids are indicative of disease which dates back at least as far as ancient Greece (Nicholson and Lindon 2008). Nowadays, metabolomics, and the related field of metabonomics, metabolic profiling, metabolic phenotyping uses modern techniques focusing on a large-scale and high-throughput measurement of small molecules in samples (< 1,500 Da, the intermediates and products of metabolism); in other words, the metabolome. These terminologies employed can become confusing due to the large number of definitions found in the literature, with the most common of these being metabolomics and metabonomics. Metabolomics refers to the study of the global, dynamic endogenous metabolic profiles in a living system (cell, tissue, or organism) under a given set of conditions, such as diseases (e.g., cancer and diabetes), pathophysiological stimuli (e.g., xenobiotics) or genetic modifications (Goodacre, Vaidyanathan *et al.* 2004). Metabonomics has been described as the "quantitative measurement over time of the metabolic responses of an individual or population to drug treatment or other intervention (Holmes, Wilson *et al.* 2008)." The focus of metabonomics is on understanding systemic change through time in complex multicellular systems, while metabolomics seeks an analytical description of complex biological samples. The difference is rather philosophical than technical, and the two terms are often used interchangeably in practice, with the same analytical and data treatment procedures (Nicholson and Lindon 2008).

The metabolic phenotype provides a readout of the metabolic state of an individual and is the product of genetic and environmental contributions under a particular set of conditions (Nicholson 2006). It has certain benefits such as integrating the different levels of genome, proteome and metabolomes, regardless of the remarkably difference in timescale. In addition, environmental factors and lifestyle have great impact on individual and population that it is far from enough to interpret their effects using gene-related data (Nicholson and Lindon 2008). Metabolic phenotyping or metabolomics can monitor the global outcome of all the influencing factors such as the variation and expression of gene (genomics), the expression and modification of protein (proteomics), and exposure to the environment, without making assumptions about the effect of any single contribution to that outcome.

Metabolomics is now establishing itself as an integral part of research efforts in the life sciences (Nicholson, Holmes *et al.* 2012, Patti, Yanes *et al.* 2012), providing us an enhanced understanding in a variety of fields including disease mechanisms, the discovery of diagnostic biomarkers, the mechanisms for drug action, drug discovery and an increased ability to predict individual variation in drug response phenotypes (Soga, Baran *et al.* 2006, Sreekumar, Poisson *et al.* 2009). It is of particular interest in areas like doping control where the metabolomics application is just emerging. Shifts in metabolomic signatures occur when drugs are administered and although these are largely unknown, once they are identified, a more sensitive and/or longer detection than the administered compound(s) can potentially be achieved. Some pilot studies concerning drug abuse in equine sports (Kieken, Pinel *et al.* 2011, Chan, Ho *et al.* 2016) and livestock production (Regal,

Anizan *et al.* 2011, Anizan, Bichon *et al.* 2012, Dervilly-Pinel, Courant *et al.* 2012) have demonstrated the possibility of applying metabolomics in the fight against doping.

The discovery of the metabolomic biomarker can be divided into two approaches, namely, targeted and non-targeted. Riedmaier et al. (Riedmaier, Becker *et al.* 2009) reviewed the potential applications of metabolomics to test steroid hormone and beta-agonist agents, and proposed the analysis of some small molecules such as glucose, fatty acid, lactate and creatinine. Many research groups have been devoted to endogenous steroid profiling using metabolomics, i.e. steroidomics (Boccard, Badoud *et al.* 2011, Kiss, Jacquet *et al.* 2011, Van Renterghem, Van Eenoo *et al.* 2011, Ponzetto, Mehl *et al.* 2016). Most of these studies are targeted metabolomics (hypothesis driven), based on the hypothesis that certain compounds or metabolic pathways may be affected by the doping substances or methods. Thus, they utilised specific methodology to match the research target.

Nonetheless, a non-targeted or "top-down" strategy is useful for finding novel biomarkers and possibly identifying new perturbed metabolism, with the aim of analysing a biofluid non-discriminately and ascertaining any differences in the concentration of the detected molecules. Non-targeted approaches are becoming more popular, with an increase in demand for hypothesis generating results. However, it is still a relatively new technique and there are certain disadvantages. Firstly, "non-target" means it tries to extract and analyse as many molecules as possible, which inevitably encounters numerous interferences. For instance, the extraction of highly abundant molecules may require a dilution step for accurate analysis, but those molecules at a low concentration will be diluted to below the

limit of detection. Other examples can be matrix effect and ion suppression during LC-MS analysis in non-targeted manner, due to the intrinsic complexity of bio-samples. Secondly, the time and economic cost can be elevated as vast quantities of information gained compared to targeted approaches. It requires comprehensive data treatment and statistical modelling to refine the information, which will be further discussed in section **1.4.1**. Additional time cost includes the identification of any features of interest. Moreover, due to the limited capacity of the database, it is very difficult to confirm the molecules in many occasions.

Despite these drawbacks, non-targeted metabolomics is gaining increasing interest. It treats the biological process as a whole without a specific focus; thus, in anti-doping field it has the potential to identify novel biomarkers and possibly unknown mechanisms or the metabolic pathways of doping agents. So far, the application of non-targeted metabolomics in anti-doping research has been rather limited (hardly any before this PhD project). Using untargeted metabolomics, Raro *et al.* recently found that 1-cyclopentenoylglycine was a potential marker for testosterone ester misuse (Raro, Ibanez *et al.* 2015); Kiss *et al.* found increased pantothenic acid (vitamin B5) in a salbutamol positive group (Kiss, Lucio *et al.* 2013). Although these findings were preliminary, they demonstrated the potential usefulness of untargeted metabolomics for doping control purposes. The M. Thevis group has been working on the application of untargeted metabolomics in doping control to investigate alternative markers for testosterone and growth hormone abuse (Thevis and Thomas 2015). Another on-going project funded by WADA has found three blood (plasma and serum) metabolites associated with red blood cells, which could be potential markers for rhEPO misuse (Pitsiladis, Gmeiner *et al.* 2016).

1.3 Analytical techniques for metabolomics

For large-scale metabolomics experiments, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the main techniques used, others such as Fourier transform-infrared (Harrigan, LaPlante *et al.* 2004, Derenne, Verdonck *et al.* 2012) HPLC-UV (Chen, Duran *et al.* 2003, Yang, Xu *et al.* 2004) approaches have also been successfully applied, though some article suggested that those approaches provide less information content in the spectrum obtained (Lenz and Wilson 2006).

1.3.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is the primary technique for global profiling as all compounds with NMR-measurable nuclei can be detected. It is a robust and reliable technique allowing the detection of a wide range of structurally diverse metabolites simultaneously (Beckonert, Keun *et al.* 2007). Based on atom-centred nuclear interactions and properties, NMR spectroscopy can provide detailed structural information of small organic molecules, therefore has enabled a large number of biofluid constituents to be identified and catalogued (Nicholson, Foxall *et al.* 1995, Lenz and Wilson 2006). Furthermore, NMR is non-invasive and non-reliance on separation prior to analysis, which enables the sample for further analysis such as GC/LC-MS. In addition, sample preparation for NMR analysis is relatively simple with little or no physical or chemical treatment required prior to analysis. As such, it is suitable for the metabolomics profiling of complex biofluid samples.

Despite being such a powerful, cost-effective and easy-applied approach, the main weakness of NMR is lack of sensitivity. This means small molecules in low abundance may not be adequately detected, thus NMR falls behind the highly

sensitive technology of mass spectrometry. However, the use of labelled compounds can compensate this aspect, leading to significant improvements in sensitivity (Putri, Yamamoto *et al.* 2013).

1.3.2 Mass spectrometry (MS)

Mass spectrometry (MS) is a pivotal tool in analytical chemistry, providing the structural characterization of biological molecules, especially those present in minute quantities in complex biological samples. MS offers qualitative and quantitative analysis of compounds with high sensitivity and selectivity (Xiao, Zhou *et al.* 2012). The recent growth of metabolomics has greatly depended on the development of mass spectrometry. The main advantages of mass spectrometry in biological analysis lie in 1) wide variety of sample type, including biofuilds and tissues, containing compounds ranging from non-polar to polar, and from low molecular weight to high molecular weight. 2) High sensitivity with tiny amount of sample used. Currently the absolute sensitivity can be up to 10^{-11}g, i.e. micro-liter sample used can achieve satisfactory results. 3) High speed of analysis as fast as 1/1000s, which is ideal for chromatograph-mass coupling technique. However, many compounds in complex mixtures give variable responses in MS experiments, which can be highly misleading (Nicholson and Lindon 2008).

Mass spectrometry can be effectively coupled to chromatographic technologies, such as LC-MS, GC-MS, CE-MS (Capillary electrophoresis–mass spectrometry) etc., thus conducting identification and quantify analysis in complex bio-samples. One advantage of LC/GC/CE-MS methods is that metabolites can be separated based on their physicochemical characteristics prior to detection and analysis,

reducing the number of molecules that enter the MS at a given time. Multiple components entering the MS at the same time can impact ionization and cause ion suppression, especially when using electrospray ionization (ESI), hence better separation tends to improve measurement sensitivity and accuracy.

In metabolomics research, LC-MS (Want, Wilson *et al*. 2010, Want, Masson *et al*. 2013) methods are predominantly used, followed by GC-MS (Chan, Pasikanti et al. 2011) and CE-MS (Garcia-Perez, Angulo *et al.* 2010) approaches, other techniques such as direct infusion MS (Southam, Lange *et al.* 2014), comprehensive GC×GC–MS (Mohler, Dombek *et al.* 2007), supercritical fluid chromatography (SFC)-MS have also been used (Jones, Rainville *et al.* 2014). HPLC-MS combines the advantage of both HPLC and MS, allowing separation of metabolites of a wide range of molecular weight and polarity with high throughput and high sensitivity. LC-MS for global metabolic profiling has been increasingly used (reviewed in (Theodoridis, Gika *et al.* 2008) (Becker, Kortz *et al.* 2012) (Fang and Gonzalez 2014)), especially those employing reverse phase (RP) separations, coupled to electrospray ionization (ESI) in both positive and negative modes of ionization, such as the profiling based on urine or plasma. Urine samples can be injected directly into the LC-MS system (Theodoridis, Gika *et al.* 2008), or more often, diluted with water before the injection onto the column (Werner, Croixmarie *et al.* 2008, Wang, Reijmers *et al.* 2009). Other protein-rich biofluids such as plasma or cerebrospinal fluids can also be analysed with minimal sample pre-treatment mainly using organic solvents to precipitate proteins (Want, O'Maille *et al.* 2006, Bruce, Tavazzi *et al.* 2009).

LC-MS was once considered unsatisfactory for the analysis of hydrophilic metabolites; however, the emergence of hydrophilic interaction chromatography (HILIC) column in recent years has successfully achieved the analysis of hydrophilic metabolites such as amino acids, organic acids, nucleic acids, sugar, sugar alcohols, sugar phosphates etc. without any derivatization (Antonio, Larson *et al.* 2008, Buszewski and Noga 2012).

Ultra-high performance liquid chromatography (UHPLC) uses even smaller particle sizes (sub-2μm) in column, and a pump with a maximum pressure of 100 MPa. It requires the solvent delivery systems capable of operating at higher pressures in the 6000–15000 psi range (Plumb, Castro-Perez *et al.* 2004). As a result, resolution, sensitivity and even speed of analysis are dramatically increased. The key chromatographic principles are as follows:

$$N = L/H$$

$$H = A + B/u + Cu \quad \text{(Van Deemter Equation)}$$

$$A = 2\lambda d_p$$

Where

- N is the number of theoretical plates, a measure of the resolving power of the column
- L is column length
- H is plate height
- A is Eddy-diffusion parameter
- B is diffusion coefficient
- C is resistance to mass transfer coefficient
- u is linear velocity
- λ is particle shape
- d_p is particle diameter

When particle size (d_p) decreases, H decreases, and N increases, leading to improved analytical performance.

The high chromatographic resolution, which leads to narrow peak widths and an increased S/N (signal/noise) compared to conventional HPLC, is advantageous in metabolic phenotyping to detect a greater number of metabolites at physiological concentrations (Dunn, Broadhurst *et al.* 2008)

The value of UHPLC-MS for metabolomics has been demonstrated in a number of studies. In a study employing rodent urine, analysis of the same set of samples by both conventional LC-MS and UHPLC-MS revealed much improved chromatographic efficiency and a higher peak count for the latter (Wilson, Nicholson *et al.* 2005). Another subsequent application showed that UHPLC with a short, 1.5 min reversed-phase gradient for a range of rodent-derived samples, gave similar performance to that was obtained using conventional HPLC-MS with a 10 min separation (Plumb, Granger *et al.* 2005).

High resolution mass spectrometry (HRMS) is a widely practiced technique in analytical and bioanalytical sciences. HRMS offers exceptionally high resolution and accurate mass measurements with sub-ppm errors, improving the sensitivity and information content of global approaches. It is thus possible to detect metabolites having the same nominal mass but distinct exact masses in complex biological matrix. It can also give the highest degree of structure confirmation, which facilitates database inquiries for identification purposes. All of these advantages make HRMS an extremely suitable tool for MS-based untargeted metabolomic experiments. HRMS has been successful applied in many metabolomic studies over the past decade, which have been reviewed in detail recently (Junot, Fenaille *et al.* 2014, Ghaste, Mistrik *et al.* 2016). Time-of-flight

mass spectrometry (TOF-MS) is a representative HRMS technique that has been widely used in metabolomic studies to obtain accurate and precise MS data (Dan, Su *et al.* 2008). TOF-MS provides high sensitivity and resolution to profile intact precursor ions which are generated from metabolites through ESI and represents the most appropriate MS instrument to apply LC separations for this objective (Mao, Yang et al. 2012, Qian, Wang et al. 2013). Fourier transform mass spectrometers (FTMS) or Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR-MS) are the most advanced mass analysers in terms of high accuracy and resolving power with sub-parts-per-million mass accuracy. The main disadvantage of FT-ICR-MS instruments, nevertheless, is their relatively slow acquisition rates (e.g. scan rate of 1 Hz with mass resolution of 100,000 at *m/z* 4000), which limits the application of FT-ICR-MS in metabolomics (Ghaste, Mistrik *et al.* 2016). Another MS technique that has been increasing used in metabolomics recent years is Orbitrap. The Orbitrap analyser provides a higher mass resolution and mass accuracy over a wider dynamic range allowing the potential detection of a greater number of metabolites of similar accurate mass, with a high level of confidence for metabolite identification (Makarov, Denisov *et al.* 2006). Similar to MS^e technology of TOF (Plumb, Johnson *et al.* 2006), Orbtirap allows the accurate mass full scan and single/multiple or full mass fragmentation spectra to be acquired simultaneously (Dunn, Broadhurst *et al.* 2008).

Generally, TOF based instruments can achieve mass resolution up to 60,000 (at *m/z* 200), while Orbitrap based instruments can achieve much higher mass resolution—up to 240,000, and over 1,000,000 for FT-ICR (at *m/z* 400) (Junot, Fenaille *et al.*

2014). However, the resolving power of TOF-MS instruments is independent of the acquisition rate, which could be considered a key feature especially when fast chromatographic separations are to be used (Hopfgartner 2011). The mass resolution of the Orbitrap is correlated to scan rate with longer acquisition time providing higher mass resolution. However, fast scan speed is preferred in metabolomics, thus the most appropriate parameters need to be chosen considering the balance between resolution and scan rate, which is studied and discussed in Chapter 3 of this thesis.

By coupling UHPLC with HRMS, various types of metabolites in a wide-ranging concentration in a complex bio-matrix can be identified effectively and efficiently. Thus UHPLC-HRMS including both TOF and Orbitrap technologies are applied in this thesis to develop a sensitive metabolomic approach to the discovery of doping biomarkers.

One drawback of these highly developed MS coupled with pre-separation techniques is the loss of information on the spatial localization of the metabolites in tissue samples. Yet recently, MS imaging (MSI) combined with soft ionization methods (e.g. MALDI, NIMS, DESI) has emerged as a technology that makes it possible to resolve the distribution of biological molecules present in tissue sections by direct ionization and detection (Miura, Fujimura *et al.* 2012), and several studies have suggested the importance of *in situ* metabolite imaging in metabolomic studies and other biomedical applications (Benabdellah, Touboul *et al.* 2009, Eberlin, Ifa *et al.* 2010, Miura, Fujimura *et al.* 2010, Greving, Patti *et al.* 2011).

Although some sophisticated analytical technologies for metabolomics with high throughput, high selectivity and sensitivity have been developed and applied for decades, a method capable of analysing all metabolites simultaneously is still currently unavailable due to the complexity and the dynamic nature of the metabolome. Therefore, researchers need to select the most appropriate approach for their work, taking account of factors such as sensitivity, resolution, throughput and cost, and ultimately multiple analytical platforms may be needed to cover the full spectrum of metabolites (Xiao, Zhou *et al*. 2012, SastiaPrama, Fumio *et al*. 2014).

1.4 Metabolomics data treatment

Metabolomics can be classified as targeted and non-targeted metabolomics as stated above, however in data treatment stage, non-targeted metabolomics are frequently followed by targeted approach focusing on specific metabolites of interest identified preliminarily using non-targeted approach, as illustrated in **Figure 1-2**.

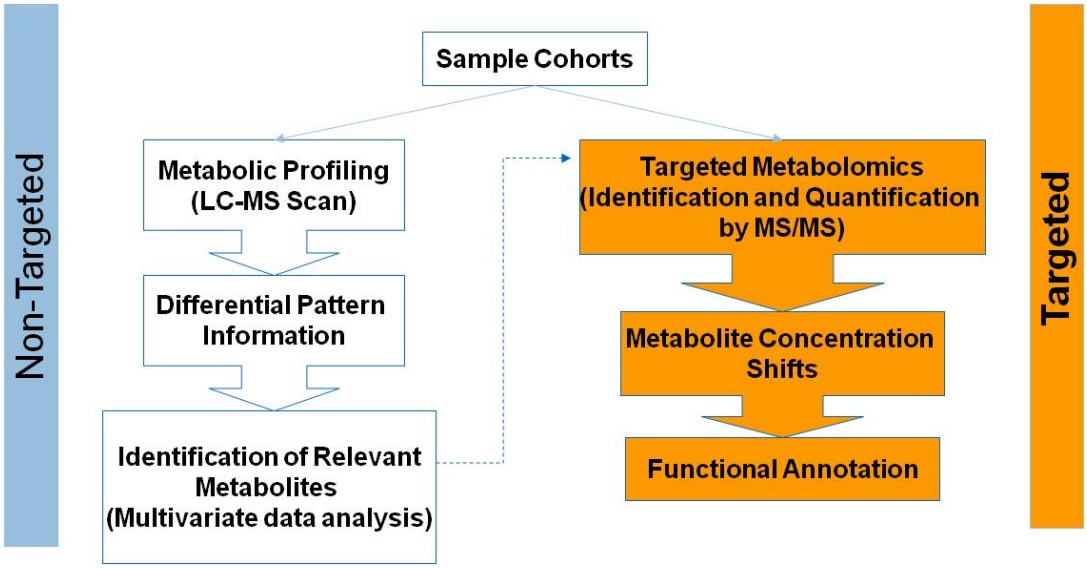

Figure 1-2 Typical workflow of metabolomics. Sample cohorts can be subjected to targeted and non-targeted metabolomics analyses. However it is also a common strategy that non-targeted metabolomics can be followed by targeted analysis once relevant metabolites are identified from multivariate analysis.

1.4.1 Non-targeted approach

Typically, a non-targeted metabolomic experiment includes a comprehensive metabolite fingerprinting step, either by NMR or LC-MS methods. The primary challenges of such untargeted experiments include detecting intact small molecules (<1500Da) over a wide concentration range, as well as ensuring sufficient metabolite coverage to shed light on the metabolic problem being investigated. Hence, non-targeted metabolomics studies often use multiple analytical technologies to study the same samples, and metabolomic protocols are now available for a variety of biofluid and tissue types. In the data pre-treatment stage, there are numerous software available for the non-targeted approach (e.g., XCMS and MZmine for LC-MS, TagFinder and metab for GC-MS and JDAMP for CE-MS etc.).

Since the data set of non-targeted metabolomics is extremely large, researchers need to focus on the metabolites relevant with the alternation of the condition. The using of multivariable analysis can help to extract the metabolites of interest from such a big data matrix. Principal component analysis (PCA) is a non-supervised chemometric method and is most widely used in metabolomic studies, visualizing the relationship among groups. The non-supervised method is to simply classify, given that sample identity is unknown. On the contrary, the supervised multivariable analysis technique such as Partial Least Squares (PLS), Partial Least Squares-Discriminant Analysis (PLS-DA), Orthogonal Partial Least Squares (OPLS), use the prior information about sample class, giving better information in biomarkers discovery (Jonsson, Bruce *et al.* 2005). Meanwhile, when searching for significant metabolites, researchers can not only use the loading matrix, but also the 'variable importance in the projection'(VIP) (Perez-Enciso and Tenenhaus 2003) and the 'coefficient variables'. Finally, the selected and calculated features (potential markers), can be subjected to database search, such as HMDB (Human Metabolome Database), Metlin, MassBank and MMCD (Madison Metabolomics Consortium Database) or more general chemical databases (e.g., PubChem or ChemSpider). The use of tandem MS (MS/MS), by acquiring MS/MS spectra of these ions, in combination of precursor *m/z* and retention time, can derive the molecular structural information, thus the results from mass-based identification can be further refined. Reference standards are normally used for the final confirmation.

An informative table describing the various forms of data treatment and chemometric approaches available in metabolomic studies was compiled in a

review by Madsen *et al.* (Madsen, Lundstedt *et al*. 2010) and can be found in **Table 1-3**.

Table 1-3 Frequently used data analysis methods in metabolomics.

Technique	Typical use	Description and comments
Univariate testing (Broadhurst and Kell 2006)	Identification of potential biomarkers	Univariate analysis where the corresponding *p*-values are the standard scientific measure of significance. Multiple testing is prone to false positives unless corrected significance limit is used
Principal component analysis (PCA) (Jackson 1991)	Data overview	Standard multivariate analysis method to provide an overview of a large dataset. Useful for identifying outliers, clusters and trends in the data. Is not a classification method
Partial least squares discriminant analysis (PLS-DA) (Wold, Sjöström *et al.* 2001)	Classification and biomarker identification	Classification variant of PLS. Works best with homogeneous classes. Biomarker identification possible, but not as straight forward as with OPLS-DA.
Orthogonal projection to latent structures discriminant analysis (OPLS-DA) (Bylesjö, Rantalainen *et al.* 2006)	Classification and biomarker identification	Classification variant of OPLS method. Ability to separate between-class from within-class variability. Straight forward interpretation and identification of potential biomarkers
Self organizing maps (SOM) (Kohonen 2000)	Clustering method	A neural network based method for identifying trends and clusters in the data. Can account for non-linearities in the data. - not there in the other descriptions.
Support vector machines (SVM) (Sitter, Bathen *et al.* 2009)	Classification (non-linear)	High flexibility in modeling non-linearities. Careful model selection reduces possibility of overfitting. Potential biomarker identification more complex compared to linear methods, except when linear variant is used (LS-SVM)

Table adapted from (Madsen, Lundstedt et al. 2010).

1.4.2 Targeted approach

After identifying the metabolites of interest by means of non-targeted statistical analysis, it comes to the step of targeted approach aiming at validation of the hypothesis or the statistical model, focusing on the objective metabolites, i.e., biomarkers. The researchers need to confer the significance and reliability of these markers. Furthermore, a highly reproducible data matrix for comparison with the previous experiments should be conducted, thus, the development of a quantifiation method is important. Normalization, by means of internal standards, external standards and total signal, should also be optimized for each research purpose. In addition, researchers can determine the optimal ion for targeted metabolite quantification to acquire highly sensitive and reproducible data.

Model construction using PLS, PLS-DA, OPLS or OPLS-DA plays an indispensable role in metabolomics research, and if the reliable and reproducible data can be obtained with the targeted approach, the robustness of the prediction model then should also be validated. Cross validation (K-fold validation) is a frequently used method for validation of the constructed model (Wold 1976, Geisser 1993, Jumtee, Bamba *et al.* 2009), with the root mean square error of evaluation (RMSEE) determined for robustness and the root mean square error of prediction (RMSEP) for reliability. Receiver operating characteristic (ROC) curve is also a commenly used strategy in the validation study of biomarkers. It investigates the accuracy of a model's ability to separate positive from negative cases (such as predicting the presence or absence of doping practice) (Lasko, Bhagwat *et al.* 2005). It is especially useful in evaluating the predictivtiy of our

models or selected biomarkers with output values over a continuous range, since it captures the trade-off between sensitivity and specificity over that range.

Based on the background mentioned above, metabolomics approaches will be applied in the field of drug control in this thesis, in order to detect surrogate markers of doping substances, and build a systematic model that can be applied in doping control.

1.5 Current biomarker research and applications in doping control

1.5.1 Anabolic-androgenic steroids

One of the first approaches used on the biomarker concept was using the ratio of testosterone (T) to epitestosterone (E) to detect the misuse of anabolic steroids in early 1980s (Catlin, Fitch *et al.* 2008). Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone, modified to enhance its anabolic actions to promote protein synthesis and muscle growth (Saudan, Baume *et al.* 2006). AAS are the most frequently detected doping substances in sports for years. According to 2014 WADA report, anabolic agent accounted 50 % of total positive findings (adverse analytical findings (AAF) and atypical findings (ATF)) (**Table 1-4**), and among the 1812 positive cases of anabolic agents, 84 % are anabolic-androgenic steroids (WADA 2015). The abuse of AAS was first witnessed in 1960s, but there was not any detection method until the implement of immunoassy (IA) in mid-1970s. However, as IA lacks of specificity, shortly GC-MS or LC-MS was required by IOC as the gold standard to test AAS. Unfortunately, GC-MS and LC-MS can only comfirm the exist of sythetic AAS, and neither of them are capable of distinguish the AAS natrully occurred in human body (such as T) from the ones

that are administrated. In 1980 Summer Olympic Games in Moscow, no positive case of AAS was reported, however rumours said the use of endogenous steroids were circulating (Catlin, Fitch *et al.* 2008). Thus intensive efforts were made by anti-doping laboratories in the following years to study the urine steroid profile. It was soon found that there is a T isomer without known biological function in human urine, namely, epitestosterone (E). The natural median ratio of urinary T to E (T/E ratio) is approximately 1:1 and the administration of T does not increase the concentration of E, thus it upsets the balance of the two steriods and increases the ratio (Donike, Barwald *et al.* 1983, Donike, Zimmermann *et al.* 1984, Catlin, Hatton *et al.* 1997). Currently a T/E ratio of greater than 4 is used as a screening indicator for possible T misuse and followed by the confirmation using isotope ratio mass sepctrometry (IRMS), which is a direct method differentiating natural and synthetic endogenous steroids by means of the ratios of stable carbon isotopes ^{13}C and ^{12}C (Becchi, Aguilera *et al.* 1994, Piper, Emery *et al.* 2011). The detection of other endogenous steroid hormones have also applied this concept such as the ratio of androsterone to etiocholanolone (Andro/Etio ratio) and Andro/T ratio (Donike, Geyer *et al.* 1992, Van Renterghem, Van Eenoo *et al.* 2010).

Table 1-4 Summary: Substances Identified as AAFs and ATFs in Each Drug Class in ADAMS (All Sports). *Table obtained from (WADA 2015)*

Table 1-4A Substances Identified as AAFs in Each Drug Class

Substance Group	Occurrences	% of all ADAMS reported findings
S1. Anabolic Agents	1479	48%
S6. Stimulants	474	15%
S5. Diuretics and Other Masking Agents	389	13%
S9. Glucocorticosteroids	252	8%
S4. Hormone and Metabolic Modulators	145	5%
S3. Beta-2 Agonists	122	4%
S2. Peptide Hormones, Growth Factors and Related Substances	91	3%
S8. Cannabinoids	73	2%
S7. Narcotics	26	0.8%
P2. Beta-Blockers	25	0.8%
M2. Chemical and Physical Manipulation	3	0.1%
P1. Alcohol	0	0%
M1. Enhancement of Oxygen Transfer	0	0%
TOTAL*	3079	

Table 1-4B Substances Identified as ATFs in Each Drug Class

Substance Group	Occurrences	% of all ADAMS reported findings
S1. Anabolic Agents	333	68%
S2. Peptide Hormones, Growth Factors and Related Substances	101	21%
Other	49	10%
S7. Narcotics	8	2%
TOTAL*	491	

ADAMS: Anti-Doping Administration and Management System. * The Adverse Analytical Findings (AAFs) and Atypical Findings (ATFs) in this report are not to be confused with adjudicated or sanctioned Anti-Doping Rule Violations (ADRVs), as the figures given in this report may contain findings that underwent the Therapeutic Use Exemption (TUE) approval process or multiple findings on the same Athlete. In addition, Atypical Findings may correspond to multiple measurements performed on the same Athlete, such as in the case of longitudinal studies on testosterone. For the more detailed definetions of AAFs and ATFs, refer to (WADA 2015).

These ratios, which are the start of the process to determine if an athelte has used AAS, however, are not always reliable as they can be influenced by a number of

factors due to existing intra- and inter- individual variabilities standing. For instance, the T/E ratio can return to baseline when the user discontinues taking T. In extreme rare cases, the athletes have naturally elevated T/E ratio. Moreover, some athletes even administrate E to lower it. Therefore, monitoring other steroid metabolites and evaluation of additional steroid ratios as potential biomarkers can possibly increase the efficiency of detecting T misuse among athletes. Numerous studies of human urinary sterioid profiles are still being carried out and the effects of envorimental and physiological factors have also been included.

Among those studies, the "-omics" strategy has been largely applied. Boccard *et al.* reported an untargeted steroidomic approach to analyse urine samples from a human clinical trial and discovered several metabolites relevant to the detection of T intake (Boccard, Badoud *et al.* 2011); Raro *et al.* used an untargeted metabolomic strategy and found 1-cyclopentenoylglycine as a potential marker for T ester misuse (Raro, Ibanez *et al.* 2015).

However, both the T/E ratio and the studies mentioned above are population based strategy. Monitoring of individual steroid profiles over time allows switching from population-based towards subject-based reference ranges for improved detection. Van Renterghem *et al*. suggested 3 ratio markers for dihydrotestosterone (DHT) and 4 ratio markers for dehydroepiandrosterone (DHEA) based on a pilot comprehensive steroid profiling on 6 subjects and reported the usefulness of subject-based referencing for the selected markers (Van Renterghem, Van Eenoo *et al.* 2010), and a similar study conducted by the same authors found 4 novel urinary steroid ratios in addition to the T/E ratio (Van Renterghem, Van Eenoo *et al.* 2011). An individual based paradiam as such can drastically increase the overall

sensitivity, and the use of multiple markers as formalized in Athlete Biological Passport (ABP) can provide firm evidence of doping with endogenous steroids, which will be reviewed in detail in section **1.5.5**.

1.5.2 Growth hormone

The abuse of growth hormone (GH) is favoured by athletes due to its anabolic, lipolytic and post-trauma healing properties, and it is a major concern of WADA and the sports regulators. It has been rumored for a long time to be used largely among athletes but for a long time there was no proper detection method (Holt and Sonksen 2008, Meinhardt, Nelson *et al.* 2010), until the direct 'isoform' approach approved by WADA from 2004 Summer Olympic Games in Athens (Barroso, Schamasch *et al.* 2009).

GH produced in the anterior pituitary is a mixture of different isoforms, of which the most abundant one is a 22 kDa protein (approx. 70%), following by the second isoform has a mass of 20 kDa (approx. 5%-10%) (Baumann 1999). GH extracted from human was previously used to treat infant growth deficiency and it is still available in black market but with the risk of transmission of Creutzfeld-jcob disease (Holt and Sonksen 2008). The recombinant human growth hormone (rhGH) was introduced in 1981, and it is composed only the 22 kDa isoform (Bidlingmaier, Wu *et al.* 2003). Thus it leads to the well-known "isoform" approach: during the administration of rhGH, the 22 kDa isoform will be abundant, whereas other pituitary isoforms will be suppressed (Wu, Bidlingmaier *et al.* 1999) (**Figure 1-3, A**). Thus, specific IA targeting these different isoforms was used based on this background. However, the specificity and the standardization of these assays

remain problematic. In addition, this approach has a limited detection window (~24 h) due to the short plasma half-life of GH (Cunha and Mayo 2002, Bidlingmaier, Suhr *et al.* 2009). Furthermore, instead of being constitutively secreted, GH is produced in a pulsatile manner and can be affected by exercise and other environmental and circumstantial challenges, which will further complicate the direct detection of rhGH misuse (Armanini, Faggian *et al.* 2002, Velloso 2008). Another shortcoming of IA approach is that it cannot identify the doping with pituitary GH and GH promotors.

All of these issues have led to the development of an indirect biomarker approach, and one approach of combined detection of insulin-like growth factor-I (IGF-I) and procollagen type III amino-terminal propeptide (P-III-NP) for testing growth hormone (GH) misuse has been approved. This strategy lies on the fact that GH action is mediated via GH-dependent growth factors such as IGF-1, IGF-binding protein3 (IGFB3) and the subunits of IFG1/IGFB3 complexes (ALS), which increase upon the GH administration. Other bone and collagen turnover markers such as P-III-NP and collagen-I telopeptides (ICTP) have also shown to increase significantly with GH administration (**Figure 1-3, B**). Intensive studies have been carried out on these markers with different designs and statistical models and finally the combination detection of IGF-I and P-III-NP was established as they have small intra-individual variability and yield the best predictive scores (Powrie, Bassett *et al.* 2007, Holt 2009, Erotokritou-Mulligan, Guha *et al.* 2012). Notably this biomarker approach can prolong the detection window up to 2-4 weeks after discounting treatment. Just before the 2012 Summer Olympics in London, this approach was approved and introduced by the WADA and has led to the detection

of a number of athletes misusing GH (Powrie, Bassett *et al.* 2007, Holt 2013), and further refinement studies are still being carried out (Holt, Böhning *et al.* 2015).

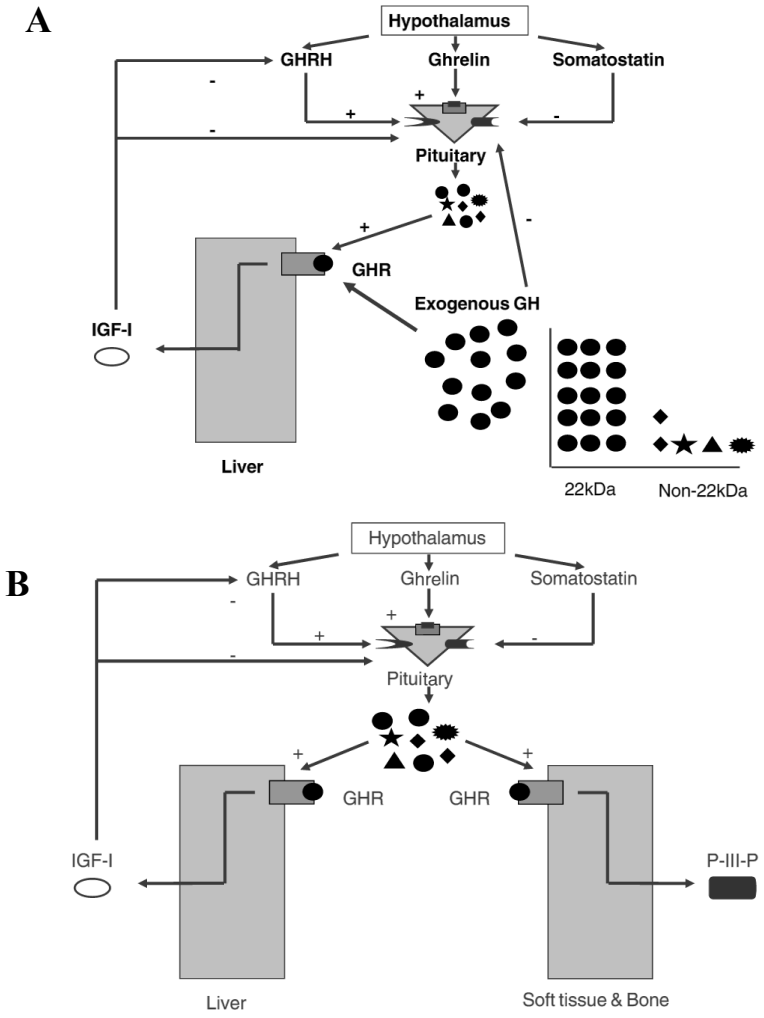

Figure 1-3 Principles of isoform and biomarker approaches to detect recombinant human growth hormone (rhGH) misuse. A, the isoform approach: under normal circumstances, GH is secreted from the pituitary under the control of the hypothalamic hormones, growth hormone–releasing hormone (GHRH), somatostatin and ghrelin. Pituitary GH appears as different isoforms, although 22 kDa represents the majority (represented in the figure as a circle). IGF-I is secreted in the liver and acts in a classical negative endocrine feedback mechanism to reduce GH secretion. The detection is based on measuring the abundance of different GH isoforms due to the suppression of natural pituitary GH caused by exogenous GH negative feedback. Consequently the proportion of total 22 kDa GH increases. B, the biomarker approach: the detection is based on measuring the GH-dependant biomarkers of IGF-1 and P-III-NP. Growth hormone acts on the liver to increase circulating IGF-I and on soft tissue to increase P-III-P. *Figure adapted from (Holt 2009)*

This biomarker approach still have some limitations such as the lack of pure recombinant IGF-I standard and differences in antibody specificity in different assays (Duntas and Popovic 2013). Despite these, there have been a number of studies focusing on finding novel biomarkers of GH.

The studies applied "omics" approaches for discovery of novel biomarkers of GH misuse are rather limited in number, mainly using genomics, transcriptomics and proteomics approaches, including applying microarray technology to detect differential gene expression profiles induce by GH (Mitchell, Nelson *et al.* 2009), quantification of circulating mRNAs for GH and growth hormone–releasing hormone (GHRH) with real-time PCR (Thakkar, Butt *et al.* 2008, Kelly, Haverstick *et al.* 2014), and proteomics-based 2-dimensional electrophoresis (2-DE) for the discovery of new GH induced serum biomarkers (Ding, List *et al.* 2009). In metabolomics field, hardly any research has been published. An LC-MS based metabolomics study assessed the metabolic perturbations caused by recombinant equine growth hormone administration in horses, and tens of ions were highlighted responsible for the modification of metabolic profile observed in treated animal, (Boyard- Kieken, Dervilly- Pinel *et al.* 2011) and this approach can possibly be extend to humans.

1.5.3 Enhancement of oxygen carriage (blood doping)

1.5.3.1 Erythropoietin

Erythropoietin is a glycoprotein produced primarily in the kidneys that acts on the bone marrow to increase erythrocyte proliferation. Recombinant human erythropoietin (rhEPO) was first produced in late 1980s to treat patient suffering

from diseases such as anaemia, a common complication of chronic kidney disease caused principally by a deficiency in the synthesis of endogenous erythropoietin. Not long after that, it was said that rhEPO were largely abused by athletes, particularly in endurance sports where increased circulating erythrocyte would enhance the oxygen carriage and consequently sports performance. It was soon banned by IOC in 1990, although no detection method was readily available at that time. In addition to that, the easy access to the substance and the enormous impact on performance resulted in widespread abuse of rhEPO during the 1990s/2000s, including the notorious case of the seven-time Tour de France winner Lance Armstrong, who subsequently admitted the use of both rhEPO and blood transfusions throughout his career.

The first direct method of isoelectric focusing (IEF) for detecting rhEPO has been available since the beginning of the 21st century. It relies on a difference in glycosylation between the endogenous and the exogenous EPO molecules, resulting in different migration characteristics during IEF. Another improved method emerged later applying SDS/SAR-PAGE (sodium dodecyl sulphate/ sarcosyl polyacrylamide gel electrophoresis). Endogenous EPO and rhEPO (and their variants) can be distinguished by SDS-PAGE as they are slightly different in their molecular mass. Other direct methods such as 2-DE, enzyme-linked immunosorbent assay (ELISA)-based methods, membrane-assisted isoform immunoassay (MAIIA) and MS methods have also been proposed as reviewed by Reichel and Pottgiesser *et al.* (Reichel 2011, Pottgiesser and Schumacher 2013). However not all rhEPO cases are detectable, because the detection window for rhEPO is known to be relatively short and, dosage and administration method

dependent (Ashenden, Gough *et al.* 2011). Furthermore, different types of rhEPO variants which are continuously produced by underground laboratories with slightly modifications from batch to batch, often do not meet the WADA positivity criteria (Pottgiesser and Schumacher 2013).

Prior to the implementation of IEF method, the indirect biomarker method had been explored for many years. In late 1990s, the so-called 'no-start' rules was introduced with the official objective to protect the health of the athletes, specifying several blood markers such as hematocrit (HCT) and hemoglobin (HGB) cannot exceed certain limits before sports (UCI 1997). These earlier markers with upper limits of definite blood values may result in athletes who would titrate or micro-dosing rhEPO to approach the HGB and HCT thresholds without exceeding them (Cazzola 2000). Another limitation is that those markers are likely to be affected by plasma volume fluctuations, which can by caused by posture, exercise, and training (Sharpe, Hopkins *et al.* 2002), altitude exposure (Ashenden, Gore *et al.* 2003, Ashenden, Sharpe *et al.* 2004), etc. To make it worse, some athletes can cover up the elevated HGB and HCT concentrations by hemodilution with intravenous infusion of saline.

Therefore, the exploration of novel biomarker of EPO has never stopped and a so-called 'ON and OFF models' strategy has been proposed (Parisotto, Gore *et al.* 2000, Parisotto, Wu *et al.* 2001). Five different serum hematopoietic parameters: [EPO concentration, soluble transferrin receptor concentration (sTFr), HCT, percentage of reticulocytes (RET %) and percentage of macrocytes] were chosen as the most clearly affected markers by the administration of rhEPO, and then multivariate models were built during current treatment (ON-models) and weeks

after withdraw (OFF-models). The sensitivity and specificity were improved by further refinement with a larger number of subjects, leading to a 'second generation' blood test (Gore, Parisotto *et al.* 2003), of which the OFF-hr model, a score combining HGB and RET %, is recommend by current ABP operating guidelines (WADA 2014). The use of indirect biomarkers of doping has culminated in the introduction of the ABP, which will be described later in section **1.5.5**.

On the other side, the "omics" strategies for identifying EPO abuse are still in their infancy, and mainly focusing on genomics and transcriptomics by analysing gene expression profile following erythropoiesis-stimulating agents (ESAs) administration, which have the similar functions of EPO to stimulate red blood cell production (erythropoeisis). Varlet-Marie *et al* identified 33 genes that could be significant markers of high dose ESA administration, and five genes showed as significant markers both in high and micro dose of rhEPO (Varlet-Marie, Audran *et al.* 2009). Further biomarkers could be derived from 'circulating miRNAs' suggested by Leuenberger *et al.* for the detection of ESAs (Leuenberger, Jan *et al.* 2011). In this context, the application of newer diagnostic tools related to the "omics" technologies including metabolomics will nurture this area in near future.

1.5.3.2 Blood transfusion

There are two types of direct blood doping, homologous blood transfusion (HBT, infusion of blood or red cell concentrates from a donor) and autologous blood transfusion (ABT, withdrawal and reinfusion of one's own blood or red cell concentrates), with the aim to directly increase red blood cells in circulation to

improve the oxygen delivery capacity. Since the IEF method for rhEPO was developed, there has been a growth in the use of blood transfusion in athletes (Soule and Lestrelin 2011).

HBT can be detected using flow cytometry, based on the fact that there are a large number of cell antigens differing from individual to individual (Nelson, Popp *et al.* 2003). While this method for the detection of HBT has been established for many years, no comparable methods currently exist for the direct detection of ABT, making it the most challenging doping method to detect.

It is worth to mention that the OFF-hr model stated above was originally developed for the detection of rhEPO, it is also sensitive to other forms of blood doping such as blood transfusion nevertheless (Mørkeberg, Sharpe *et al.* 2011, Pottgiesser, Sottas *et al.* 2011). As such, ABT is currently monitored using indirect method of markers in a longitudinal profile included in haematological module of ABP (see section **1.5.5**). However micro-dosing still remains undetectable (Ashenden, Gough *et al.* 2011), and suspicious profiles in athletes often result from altitude, heat stress or illness (Salamin, De Angelis *et al.* 2016). Therefore additional biomarkers are needed.

Salamin *et al.* recently reviewed the emerging biomarkers for ABT, which are developed based on "omics" approaches as well as plasticizers in urine (Salamin, De Angelis *et al.* 2016). Reliant on transcriptomics, ABT altered gene expressions were identified using digital multiplexed gene expression, and it was found that those genes are related to red blood cell metabolism (Salamin, De Angelis *et al.* 2016). Also, blood reinfusion can cause a distinct change in the pattern of

circulating miRNA, which is detectable up to 3 days post-transfusion. Ironomics found ferritin and hepcidin (a hepatic peptide that regulate the availably of iron to erythropoiesis) can serve as potential biomarkers of ABT (Leuenberger, Barras *et al.* 2016). The changes of red blood cells in protein level during the storage can be identified by proteomics approach, for instance Peroxiredoxin 2 was preliminary identified as potential markers of blood transfusion (Rinalducci, D'Amici *et al.* 2011, Marrocco, Pallotta *et al.* 2012). It is concluded that the "omics" technologies can provide a variety of promising markers which could be tested in an individual based model, with the potential to largely improve the ABP approach (see section **1.5.5**).

In 2010, a stratgy of di-(2-ethyhexyl) phthalate (DEHP) and its metabolites as indirect markers of ABT was proposed (Monfort, Ventura *et al.* 2010). DEHP is a plasticizer contained in blood bags acting as a protective role on erythrocytes survival and, can infuse into blood duing long-term storage. When the blood is reinfused into the body, DEHP will circulate in the body and its metabolites can be detected in urine by LC-MS/MS (Monfort, Ventura *et al.* 2012). Notably the phthalates method is the only approach proposed so far to detect blood transfusion using urine as analysis matrix, which could be easily collected in anti-doping controls.

Figure 1-4 summrizes the current and potential detection methods for the enhancement of oxygen carriage (blooding doping), including rhEPO, HBT and ABT.

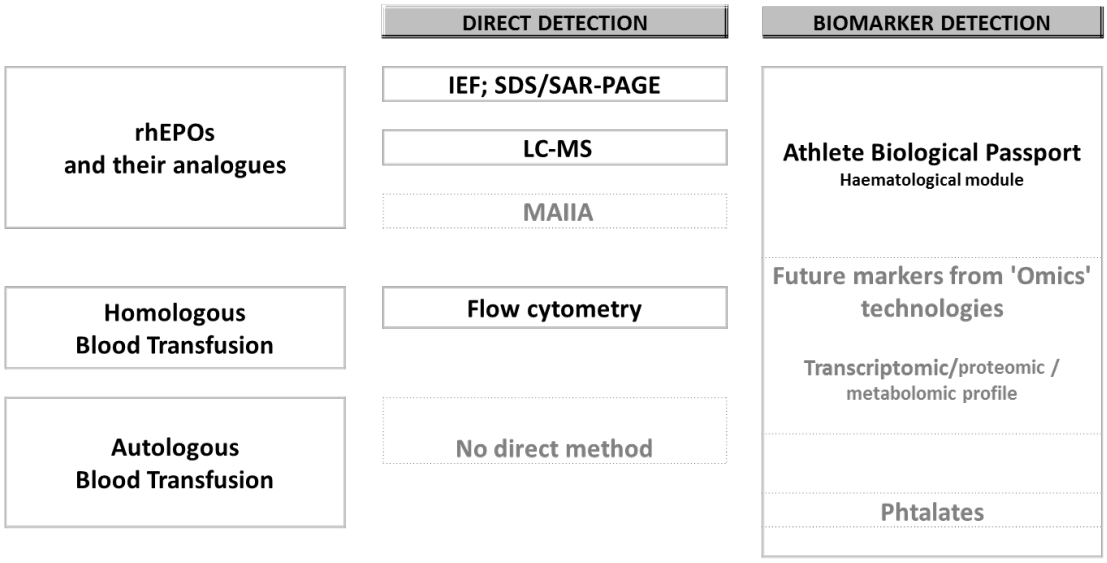

Figure 1-4 Summary of established and potential detection methods for blood doping, grouped by direct detetion and biomarker detection. Established methods are printed in black (solid line), potentail or experimental methods are printed in gray (dashed line). rhEPOs, recombinant erythropoietins; IEF, isoelectric focusing; SDS/SAR-PAGE, sodium dodecyl sulphate polyacrylamide/ sarcosyl polyacrylamide gel electrophoresis; MAIIA, membrane-assisted isoform immunoassay. *Data obtained from (Reichel 2011, Pottgiesser and Schumacher 2013, WADA 2014)*

1.5.4 Gene doping

Gene therapy has made great progress in the last decade. It attempts to treat patients by direct introduction of genetic material into the body or by up-/down-regulation of the activity of some genes. *In-vitro* and animal studies have proved the possibility of gene doping via the improved production of human recombinant proteins, such as GH, IGF-1 and EPO, and a few therapeutic clinical trials have showed encouraging results (Lee, Barton *et al*. 2004, Khan, Brown *et al*. 2005, Haisma and de Hon 2006, Hojman, Gissel *et al*. 2007, Brzezianska, Domanska *et al*. 2014). Unfortunately, this technique is inevitably pursued to be misused in sports with the attempt to create a "super althlete".

By its nature gene doping cannot be controlled by the application of technologies currently in use in anti-doping laboratories, thus there is still no completed work on the standardization of reliable tests to detect gene doping and scientists are making a lot of efforts to develop novel detection methods.

Direct methods development are mainly focusing on detecting the gene carrier (usually a vector) and the transgenes using PCR-based assays, as reviewed by Perez *et al* (Perez, Le Guiner *et al*. 2013). The methodology for detecting EPO gene doping, involving the PCR assays targeting intronless complementary DNA (cDNA) sequences that are present in doping genes but absent in intron-containing endogenous genes, is under implementation in WADA accredited laboratories (WADA 2016).

Observing the host immune response to the viral vectors via measuring the parameters such as antibodies to the viral in blood can be used as an indirect method to identify gene doping. However this method is often unable to discriminate between natural infection and artificial introduction of the virus and, with genetically engineered viral vectors which are less immunogenic, the immune response could be minimized (Baoutina, Alexander *et al*. 2008, Brzezianska, Domanska *et al*. 2014).

Apart from monitoring the immune response, indirect evaluation of changes of markers in gene, protein and metabolite patterns using genomics/ transcriptomics, proteomics and metabolomics is trending in gene doping control research, as reflected from a number of WADA funded projects (WADA 2016).

Transcriptomics/ genomics, which is capable to identify subtle changes in thousands of genes using microarray technology, can assess the expression profile of endogenous genes that may be modified following the expression of the introduced genes (Baoutina, Alexander *et al*. 2008). Proteomic profiling for detection of gene doping is based on the structural difference between the recombinant proteins produced by the transgenes and the endogenous counterparts, which can be identified using SELDI-TOF (surface enhanced laser desorption/ionization time-of-flight mass spectrometry) (Issaq, Veenstra *et al*. 2002). Metabolomics, while analysing small molecule metabolites, could provide evidence for suspicious metabolic responses to these artificial stimuli, however no study has been reported so far applying metabolomics strategy to identify gene doping.

Alternatively, ABP has the potential to address the gene doping of different gene targets, which will be discussed in the following section.

1.5.5 The Athlete Biological Passport (ABP)

The introduction of the Athlete Biological Passport (ABP) is considered a milestone in the anti-doping movement over the years. On top of historically directly detecting an administered prohibited substance or its metabolites, authorities now can longitudinally monitor selected biomarkers that would indirectly reveal the effects of doping in individual athlete through the implementation of the ABP programme. In addition to population-based parameters, this paradigm focus more on the subject-based fluctuations compared with their own historical, intra-individual data (Saugy, Lundby *et al.* 2014). The

ABP intends to establish that an athlete is manipulating his/her physiological variables, without necessarily relying on the detection of a particular prohibited substance or method using traditional targeted analytical approaches. The ABP does not replace traditional testing methods, but rather a complementary strategy to further refine and strengthen overall anti-doping programmes. The intelligent, timely interpretation of ABP data can be used to identify athletes for specific targeted testing, and may also be used to pursue an ADRV in the absence of a positive analytical test (Adverse Analytical Finding, or AAF) in accordance with World Anti-Doping Code (WADA 2014). The ABP introduces a new form of doping evidence and, as such, paves the way for a more global and integrated fight against doping. At present, ABP includes a haematological module and a steroidal module, which use statistical algorithms and in-depth expert analysis to determine the likelihood that a fluctuation in an individual athlete's passport is indicative of hematologic or steroid doping (Sottas, Robinson *et al.* 2011).

The haematological module has been in place in some federations since 2008, with the intention to gather the information on the potential markers for blood doping, such as rhEPO doping, blood transfusion and gene doping. The module was developed based on the model previously mentioned in section **1.5.3.1**, and has been continuously appraised, leading to subsequent markers and measurements added in. Currently in this module, ten markers affected by blood doping have to be measured and longitudinally recorded: HCT, HGB, red blood cells count (RBC), RET %, reticulocytes count (RET#), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (standard deviation) (RDW-SD) and immature

reticulocyte fraction (IRF) (WADA 2014). Further calculated markers specific to the haematological module include the aforementioned stimulation index OFF-hr Score (OFFS), which is a combination of HGB and RET % (Gore, Parisotto et al. 2003), and Abnormal Blood Profile Score (ABPS), which is a combination of HCT, HGB, RBC, RET %, MCV, MCH, and MCHC (Sottas, Robinson et al. 2006). The intra-individual variabilities of these blood markers are lower than their corresponding inter-individual variabilities (Sharpe, Ashenden et al. 2006, Sottas, Robinson et al. 2008), and each athlete has his/ her own reference values of the markers. The Union Cycliste Internationale is the first organization to implement the haematological module of the ABP in 2008, to identify blood doping in elite cycling (**Figure 1-5**), and subsequently several riders have been prosecuted and sanctioned on the sole basis of their abnormal haematological profiles (Sottas, Robinson et al. 2011).

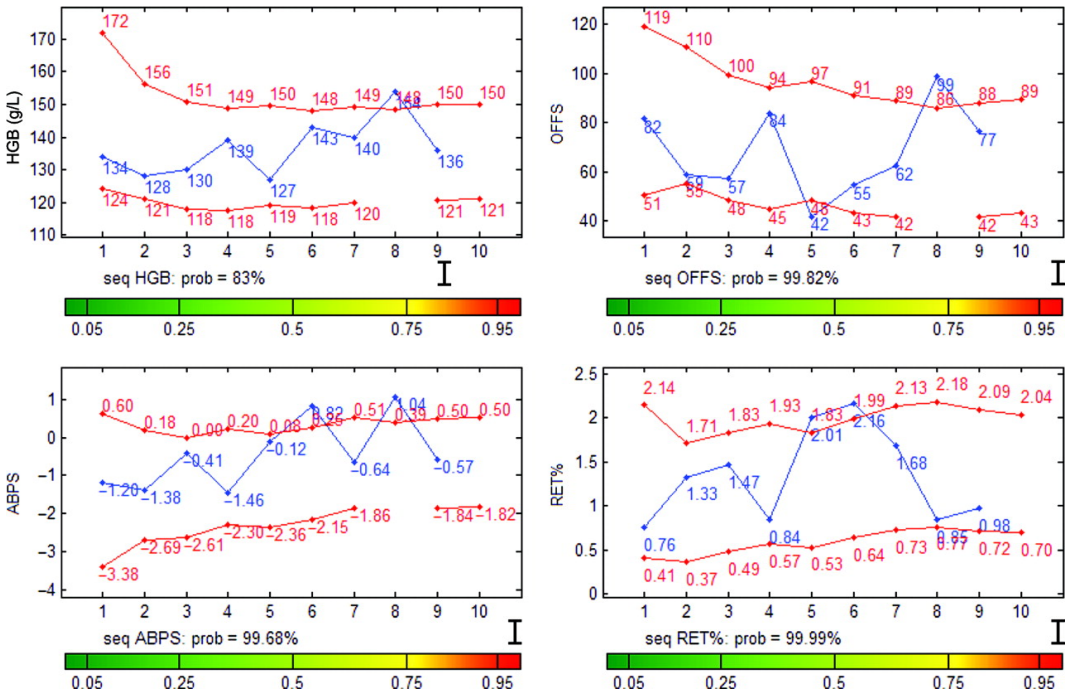

Figure 1-5 Example haematological passport of an elite rider. The figure included tests on 9 occasions for 4 markers of blood doping: haemoglobin (HGB), the stimulation index OFF-hr Score (OFFS), the Abnormal Blood Profile Score

(ABPS), and the percentage of reticulocytes (RET %). Blue lines represent actual test results. Red lines indicate limits at which the test result is considered abnormal. Initial limits (e.g., 124–172 g/L for HGB) are based on population epidemiology and are adapted in the course of individual data acquisition to produce individual final limits (121–150 g/L for HGB). Colour bars indicate sequence (seq) abnormality (Sottas, Robinson *et al.* 2010); prob, probability. The numerous abnormalities suggest that it is very unlikely that such a blood profile would not be obtained under normal physiological conditions. The rider was subsequently convicted of doping with the ESA known as CERA (continuous erythropoiesis receptor activator). *Figure obtained from (Sottas, Robinson et al. 2011)*

The steroidal module took effective in January 2014, aiming to identify endogenous anabolic androgenic steroids (EAAS) when administered exogenously and other anabolic agents, such as selective androgen receptor modulators (SARMS). The steroidal marker in urine includes T, E, T/E ratio and four testosterone metabolites, androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol). Further urinary ratios of steroid metabolites to be considered include A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E (WADA 2014).

The indirect markers in the ABP can be affected by the heterogeneous and confounding factors (Sottas, Robinson *et al.* 2010), thus additional individual athlete information for the ABP is also required to provide as precisely as possible for a better interpretation of the markers, which includes (but not limited to) sex, location of testing, type of sports, whereabouts information, recent blood loss or gain, recent training and competition schedules, exposure to extreme heat conditions, usage of hypoxic devices, and altitude information (Lippi and Franchini 2010, Lundby, Millet *et al.* 2012, WADA 2014). Among those, training at high altitude is considered one of the factors that can cause the highest variability of the model in the haematological module, as a result it is generally accepted that

exposure to high altitude during the two weeks preceding the sample collection must be taken into account in establishing individual limits (Sottas, Robinson *et al.* 2010). The steroidal ratios are robust and do not change due to circadian rhythm or physiological conditions such as exercise workload, but they can be largely affected by heterogeneous factors such as sex and age (Mareck, Geyer *et al.* 2008, Saugy, Lundby *et al.* 2014).

Although at the moment longitudinal monitoring exists only for hematologic and steroidal variables, the third module of ABP, namely endocrinological module is currently underway to be finally structured and validated (Saugy, Lundby *et al.* 2014, Holt, Böhning *et al.* 2015), aiming to detect GH axis misuse such as GH, IGF-1 and GHRH, based on the aforementioned GH dependant biomarkers, IGF-1 and P-III-NP. With the longitudinal sequence of those markers, the implementation of the endocrine module in the ABP can undoubtedly provide more selective evidence to prove manipulations of growth promoters.

ABP still need further refinement as the sensitivity of the ABP to detect doping is limited if the physiological result of a low level of doping remains within the individual's own reference range, for instance it cannot flag the EPO micro-dosing at this time (Ashenden, Gough *et al.* 2011). Thanks to the recent development of high-throughput "omics" technologies, they can provide new opportunities to discover biomarkers for the ABT. Each doping violation has its own pattern in proteomic or metabolomic profile, therefore a high specificity will be achieved by the recognition of a unique signature in high-multidimensional profiles derived from "longitudinal–omics", instead of a single biomarker with supposedly high specificity to doping (**Figure 1-6**) (Sottas and Vernec 2012). Transcriptomics,

proteomics and especially metabolomics have the potential to significantly strengthen the ABP approach and contribute to other traditional anti-doping tests, by providing a single proteomic or metabolomic profile, allowing the detection of large cohorts of substances. The identified specific fingerprints in this way could be integrated in the ABP following due validation and assessment of confounding factors (Vernec 2014).

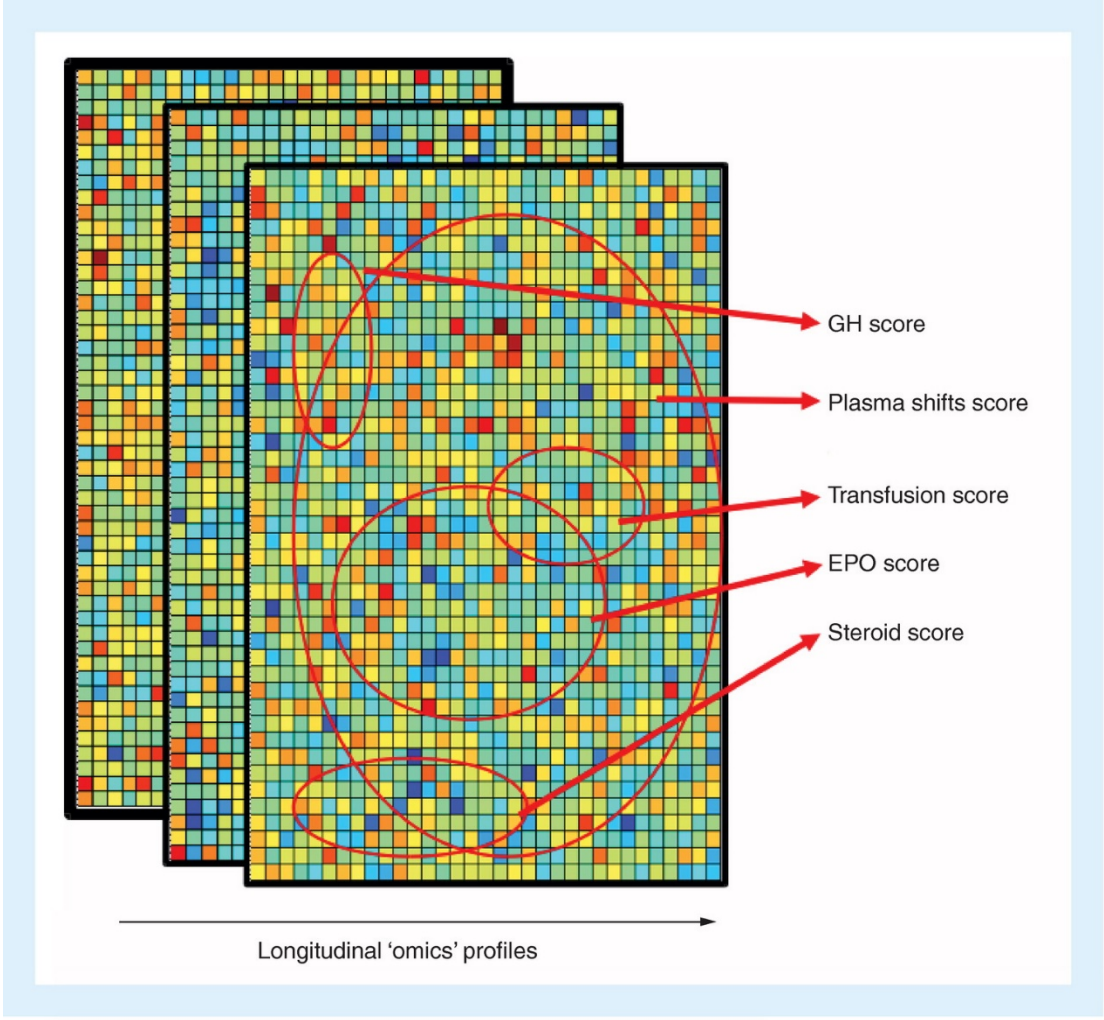

Figure 1-6 Possible "longitudinal-omics" collated in an Athlete Biological Passport to detect doping "fingerprints". Each colour map represents the athletes' "omics" data at a particular time. Each small coloured square within a map represents a feature measured in the "omics" field (e.g., a metabolite in metabolomics or a protein in proteomics) with the colour indicating the measured level in a biological fluid. Red represents a high value for this particular subject, blue a low value for this subject. In a non-targeted approach, the detection method

is based on the recognition of "omics" patterns specific to the doping substance: the wider the profile, the more likely the tests would detect a specific doping pattern. *Figure obtained from (Sottas and Vernec 2012)*

1.5.6 Limitations of the biomarker approach

The main advantages and disadvantages of direct and biomarker approaches are outlined comparatively in **Table 1-5**:

Table 1-5 The main advantages/disadvantages of direct vs. biomarker approaches

	Advantages	Disadvantages
Direct method	• Can be easily validated and accepted • Reliable	• Not able to address the emerging threat (e.g. designer drug) • Difficult to detect endogenous substance or biological technologies • Time consuming • Limited detection window • Expensive
Biomarker method	• Able to detect endogenous doping substance • Downstream biomarkers rather than the agent itself • Could have a longer detection window • Intelligence-based complementary approach for fast screening	• Difficult to validate and can get challenged • Can be affected by confounding factors • Variabilities exist, not always reliable • Need direct method as final confirmation method in most cases

Since the biomarker approach is an indirect method to screen for agents based on their physiological effects rather than their structure, validation is still the major challenge in terms of assuring an accreditation body of its effectiveness, and this limits the use of this biomarker approach to confirm the misuse of an agent or

method. Moreover, biomarkers applied to sports testing need to be highly discriminatory to meet the forensic standards compared to clinical diagnose. To this end, large-scale validation studies are needed to consider various circumstances and provide sufficient evidence for a legally binding indirect test.

The diversity of the population has always been an issue when applying a biomarker, making it difficult to determine of the abnormal limits. The ABP programme with the longitudinal monitoring of individual subjects can overcome this problem and encouragingly, this has been fully accepted by the Court of Arbitration for Sport (CAS) in recent hearings (Saugy, Lundby *et al.* 2014).

However, this promising tool reaches its limit when confounding factors are used to justify the abnormal values of the markers, such as the effect of recent training at a high altitude in ABP haematological module. Indeed, the huge intra-individual biological variation caused by exercise, diet, or environmental factors could lead to physiological changes in the levels of RNA, protein and metabolites. These various confounding factors and their effect should be addressed by studying sufficiently large and heterogeneous populations in different confounding circumstances during the development phase of the biomarker method in order to ensure the specificity of a biomarker test. However it is still very difficult to assess the impact of environmental factors such as seasonal and temperature effects because of the high frequency of travel of athletes across different continents, despite the use of the "Whereabouts" system (Delanghe, Maenhout *et al.* 2014). Another hurdle is that ordinary members of the population cannot totally replace élite athletes in studies because of their physiological differences and this may lead to legal and ethical

concerns that could constrain the latitude of the experimental design (Teale, Barton *et al.* 2009).

Large scale "omics" approaches can respond to the need for more and more novel biomarkers to enhance the sensitivity when confronted with sophisticated low-dose doping schemes, such as the failure to detect multi-level micro-dosing, as discussed in section **1.5.5**. Nonetheless, although "omics" approaches have been used in the discovery of indirect biomarkers for doping for the last decade, the results have not yet led to any routine application in anti-doping control. This is mainly due to the biological complexity and inter-individual variation, as well as the intricate validation procedures mentioned above.

The practical limits of these methods include the frequent and regular profiling of a single athlete, with consequential practical and laboratory limitations. Other factors, including various sample collecting strategies and inter-laboratory variations can reduce the efficiency of the biomarker approach, especially the ABP; thus, the standardisation is mandatory from sampling conditions to analysis (Delanghe, Maenhout *et al.* 2014, WADA 2014).

1.6 Dried biofluid spots (DBS)

1.6.1 DBS and their practical applications

Dried biofluid spots (DBS) are obtained by spotting small volumes (typically less than 20 μL) of biofluids, such as blood, urine, cerebrospinal fluid (CSF) and bile specimens onto different types of absorbent paper. DBfdaS have been used in preclinical drug development, disease diagnostics and treatment since the 1960s (Naylor and Chace 1999, Wilcken, Wiley *et al*. 2003, Barfield, Spooner *et al*. 2008, Turpin, Burnett *et al*. 2010, la Marca, Giocaliere *et al*. 2012, la Marca, Malvagia *et al*. 2012, Demirev 2013, Jiang, Sidhu *et al*. 2016), and DBS studies are also becoming increasingly popular in drug analysis (Barfield, Spooner et al. 2008, la Marca, Malvagia et al. 2008, Abu-Rabie and Spooner 2009, Spooner, Lad et al. 2009, Van Berkel and Kertesz 2009).

DBS, especially dried blood spots have begun to attract increasing interest recently for their potential in anti-doping analyses. Compared to a conventional sampling collection technique, DBS offer benefits that include minimal invasiveness and easier storage and transport. Further advantages are longer stability, the possibility for automation, and robustness against manipulation, thereby constituting a promising matrix for anti-doping purposes (Ganz, Singrasa *et al*. 2012, Martin and Cooper 2014, Sadones, Capiau *et al*. 2014, Wagner, Tonoli *et al*. 2016). The detection of growth hormone, IGF-1, rhEPO and blood transfusion as well as the hematological module of ABP currently rely solely on blood specimens. However, despite an increase in recent years, there are much fewer blood samples than urine samples being collected, mainly because of the strict blood collection protocols

(e.g. the necessity of the presence of a licensed phlebotomist supervised by a doping control officer and the need to dispatch samples in a refrigerated state as soon as possible after collection, ideally arriving at the accredited laboratory on the same day and preferably within 36 h of collection (WADA 2016)). Therefore, the substantial expenditure and logistical difficulties that limit the time and location of the sample collection could be alleviated by using DBS. Although DBS have not been implemented in routine doping control analyses, some inspirational applications have demonstrated the suitability for using DBS in anti-doping tests (Peng, Segura *et al*. 2000, Cox, Rampton *et al*. 2013, Tretzel, Thomas *et al*. 2014, Reverter-Branchat, Bosch *et al*. 2016, Thevis, Geyer *et al*. 2016, Tretzel, Gorgens *et al*. 2016).

The small sample volume of typically 20 μL of blood requires high sensitivity of the detection instruments, thus the mass spectrometer are mostly used, nonetheless, other analytical methods such as immunoassays for GH have also been developed (Reverter-Branchat, Bosch et al. 2016).

1.6.2 DBS in metabolomics

The use of diverse analytical approaches in large-scale metabolomics studies emphasises the need for standardised and robust sample storage methods. At the discovery stage of metabolomics, the use of small amounts of biofluids also confers several advantages and is the ethically-preferred route (particularly when dealing with infants and small animals). In addition to the small sampling volume, DBS have a number of other practical benefits, as mentioned above; thus, it is an appealing sampling matrix for large-scale metabolic profiling. Therefore, spot

profiling can result in a more economical experimental design, particularly when considering the cost of animals, sampling, storage and shipment. Moreover, if DBS can be implemented in doping control, the DBS collected from routine tests would be very attractive samples for metabolomics and proteomics research in the discovery of novel doping biomarkers.

Pioneering metabolomics studies have shown the utility of DBS fingerprinting with ^{1}H NMR spectroscopy, UHPLC–MS and GC–MS-based methods (Constantinou, Papakonstantinou *et al.* 2004, Michopoulos, Theodoridis *et al.* 2010, Kong, Lin *et al.* 2011). The cited UHPLC–MS investigation was not limited to blood, but also showed that urine and serum spotting are viable alternatives when directly compared with classic metabolite profiling and data treatment. In their study, Michopoulos *et al.* proved that best results are achieved when spots are stored frozen on additive-free collection cards, since additives can interfere with the quality of the raw data (Michopoulos, Theodoridis *et al.* 2010, Michopoulos, Theodoridis *et al.* 2011). Another study validated a novel lipid profiling method based on HRMS using dried blood spots and concluded that, although spotting and drying had notable effects on some of the lipids, it was still possible to obtain information on changes in the global lipid metabolism. They also pointed out that the results from DBS were no less reproducible than other lipid profiling methods that required comparatively larger samples (Koulman, Prentice *et al.* 2014).

Hence an in-vial spot extraction and UHPLC-MS analysis method for metabolomics was first developed and validated in this thesis (see Chapter 2). This work combines the benefits of a spot analysis with the metabolic coverage and analytical reproducibility of the in-vial extraction (IVDE) method for

metabolomics. Further assessments include a comparison of the novel method and the conventional method, as well as the short-term storage stability of urine specimens (see Chapter 4).

The overall hypothesis of this thesis is that LC-MS based metabolomics have the potential to discover the surrogate biomarkers in doping. To test this hypothesis, 6 following chapters will be conducted. In short, Chapter 2 will explore the analytical method-development in metabolomics, overall assessing the amount and quality of the metabolic information acquired in small sample volumes using paper spots. Chapter 3 will develop methods for high resolution and high mass accuracy instrument to aid with the identification of molecules. Chapter 4 will bring the work performed in chapters 2 and 3 together, by evaluating dried urine spot metabolomics using high-resolution mass spectrometry. In chapter 5 and 6, the metabolomics strategy will be applied to actual doping samples to validate the hypothesis. Finally, Chapter 7 will summarize all the findings and evaluate how the evidence supports the hypothesis. A schematic flow and interaction of the chapters is described in **Figure 1-7**:

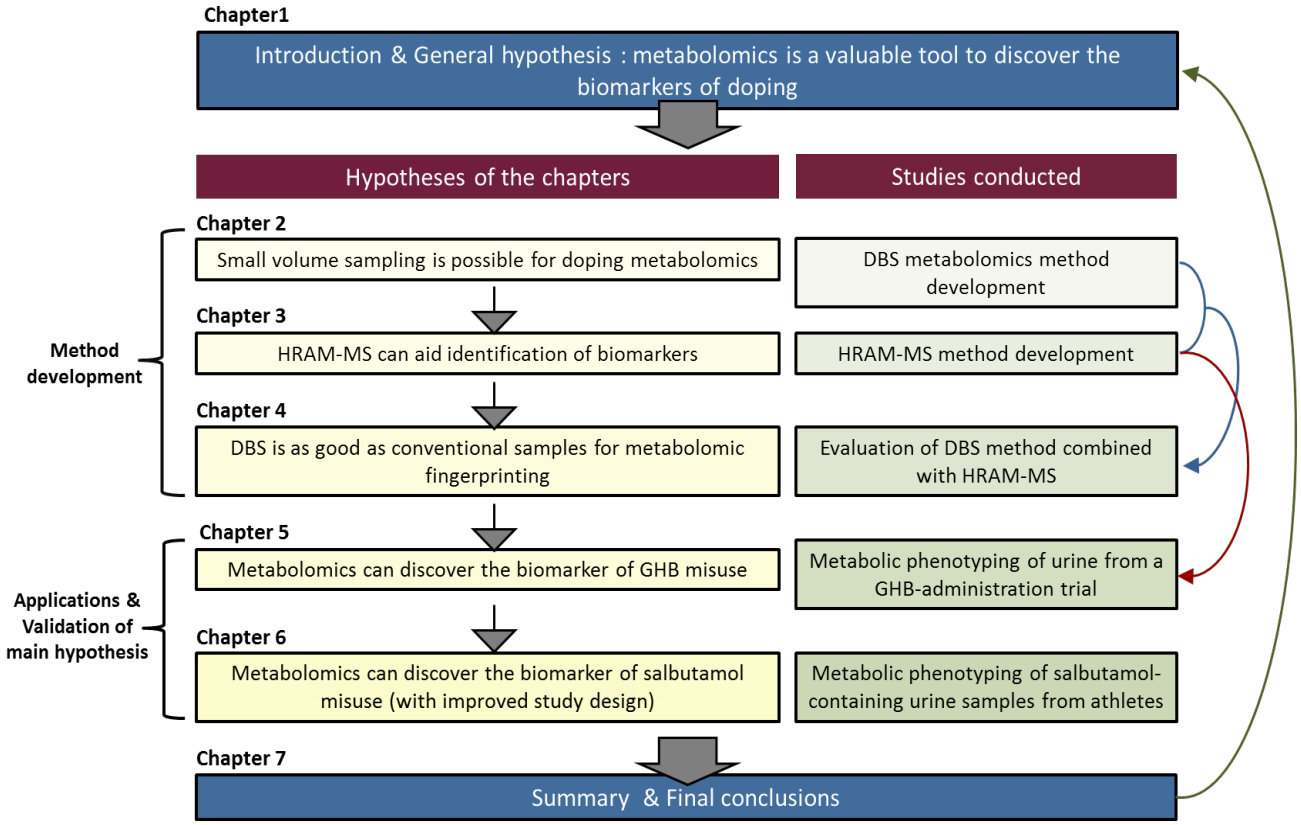

Figure 1-7 The schematic flow and interaction of the chapters.

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Chapter 2 The feasibility of paper spots for metabolomics: Metabolic phenotyping of the healthy rodent model using in-vial extraction of dried serum, urine and cerebrospinal fluid spots

2.1 Introduction

Metabolomics-based studies have already contributed to major advances in biomedical knowledge: e.g. the correlation of diet and gut microbial activities with high blood-pressure using NMR metabolomics (Holmes, Loo *et al.* 2008), and the association of gut flora metabolism with cardiovascular disease through LC-MS based metabolomics (Wang, Klipfell *et al.* 2011). However, as an "omics" approach it is not as established as other methods such as proteomics and genomics, in particular since it deals with the small-molecule fingerprinting of complex biological samples and aims to identify metabolites in samples containing a multitude of similar molecules.

Typically, an untargeted metabolomics experiment includes a comprehensive metabolite fingerprinting step, either by NMR or LC-MS methods. The primary challenges of such untargeted experiments include detecting intact small molecules (<1500 Da) over a wide concentration range, as well as ensuring sufficient metabolite coverage to shed light on the metabolic problem being investigated. Hence, untargeted metabolomics studies often use multiple analytical technologies to study the same samples, and metabolomics protocols are now available for a variety of biofluid and tissue types. For large-scale metabolomics experiments, LC-MS (Want, Wilson *et al.* 2010, Want, Masson *et al.* 2013) and NMR (Beckonert, Keun *et al.* 2007) methods are predominantly used, followed by GC-MS (Chan, Pasikanti *et al.* 2011) and CE-MS (Garcia-Perez, Angulo *et al.* 2010) approaches.

The diversity of analytical approaches used in large-scale metabolomics studies emphasizes the need for standardized and robust sample storage methods. At the

discovery stage of metabolomics, the use of small amounts of biofluids also confers several advantages, and is the ethically preferred route (particularly when dealing with animal models) (Burnett 2011) One small-volume sampling approach involves profiling dried blood spots (DBS), blood samples which have been collected onto different types of absorbent paper. Such spots have a number of practical benefits, including the added ease of storage and transportation at ambient conditions. As stated in the section **1.6.1**, dried spots have been widely used in many fields such as preclinical drug development, diagnostics, treatment and drug analysis for more than 50 years. Pioneering metabolomics studies have shown the utility of DBS fingerprinting with ^{1}H NMR spectroscopy, UHPLC–MS and GC–MS based methods (Constantinou, Papakonstantinou *et al.* 2004, Michopoulos, Theodoridis *et al.* 2010, Kong, Lin *et al.* 2011). The UHPLC–MS investigation cited was not limited to blood, but also showed that urine and serum spotting are viable alternatives when compared directly with classic metabolite profiling and data treatment. Another study by Michopoulos *et al.* proved that best results were achieved when spots are stored frozen on collection cards which are additive-free, as the latter could interfere with the quality of the raw data (Michopoulos, Theodoridis *et al.* 2010, Michopoulos, Theodoridis *et al.* 2011). Spot profiling can thus result in a more economical experimental design, particularly when considering the cost of animals, sampling, storage and shipment.

Whilst our laboratory have previously succeeded in devising a small volume (20 μL) plasma fingerprinting method that yields comprehensive metabolite coverage (Whiley, Godzien *et al.* 2012), optimising coverage per volume or mass unit of sample is also critical in metabolomics. In this chapter, a method for biofluid spot

sample preparation, in-vial and in-vial dual extraction (IVDE), and UHPLC-MS analysis for use in metabolic fingerprinting is described. We have investigated the robustness and capabilities of this method for metabolite phenotyping by testing it on rat serum and RPMI cell nutrient broth analysis and have applied the method to the study of serum, urine, and CSF from two separate rodent (rat and mouse) models. As previously demonstrated, the IVDE method yields reproducible data with low analytical variation, and requires very small amounts of biofluid for successful implementation. The aim of the study described herein thus is to combine the benefits of spot analysis with the analytical reproducibility of the in-vial extraction (IVDE) method for metabolomics, which can give a viable alternative for anti-doping metabolomics.

2.2 Methods

2.2.1 Chemicals reagents and materials

LC-MS grade water and methanol, analytical-grade formic acid and methyl *tert*.-butyl ether (MTBE), as well as rat serum used for validation experiments, were purchased from Sigma-Aldrich (UK). Samples were spotted onto Whatman FTA® DMPK-C Cards from GE Healthcare Bio-Sciences (NJ, USA). All sample extractions were performed in amber glass HPLC vials with fixed 0.3 mL inserts (from Chromacol: Welwyn Garden City, UK). RPMI-1640 cell nutrient broth was purchased from Invitrogen.

2.2.2 Rodent biofluid harvesting and spot preparation

Three adult male and three adult female Sprague–Dawley (SD) rats (250–280 g), and three normal c57 mice (25 g) were obtained from the Laboratory Animal Unit of the LKS Faculty of medicine in the University of Hong Kong, and were maintained in a temperature-controlled room with a 12 h light/dark cycle. The animals were handled according to protocols approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. The rats were anesthetized with an intra-peritoneal injection of a ketamine/xylazine mixture (ketamine 80 mg/kg and xylazine 8 mg/kg; from Alfasan: Woerden, Holland). The skin at the back of the neck, on the chest and the lower abdomen was shaved, and alcohol pads were used to clean the skin. A horizontal incision opened the lower abdominal wall and a 1 mL syringe was used to withdraw urine directly from the bladder. A longitudinal incision in the back of the neck skin was then made, through which the foramen magnum was found. A 1 mL syringe was used to puncture through the dura and withdraw CSF. Finally, through an incision in the chest, blood was withdrawn from the right atrium using a heparinized 5 mL syringe. The blood samples were centrifuged at 4000 r.c.f. for 20 min at 4 ˚C and plasma was collected.

Spots were prepared by dropping 15 μL of each biofluid onto sample spots on Whatman FTA® DMPK-C cards, with the exception of murine CSF, which was diluted 10x in MilliQ water before spotting. Between 2 and 4 replicates were prepared for each biofluid type per animal. Spots were air-dried for two hours at room temperature and then placed in PE plastic packaging for shipment. Samples were shipped in the absence of desiccant, at ambient temperature. A total of 30 spots bearing murine biofluids (urine = 12, serum = 12, CSF = 6) and 47 spots

bearing rat biofluids (urine = 12, serum = 22, CSF = 13) were extracted and analysed. Samples for validation were prepared by creating spots with 10 µL commercially obtained rat serum (x10) and 10 µL RPMI1640 (cell nutrient fluid) (x10). Validation samples were prepared to investigate analytical reproducibility of the spot-IVDE methodolog at 10 µL volumes. Mice and rat biofluids were 15 µL in keeping with conventional blood collection volumes (max 0.6 ml/kg/day).

2.2.3 Sample spot extraction

The extraction methods were adapted from a procedure previously used for the analysis of plasma by Whiley *et al*.(Whiley, Godzien *et al.* 2012) Spots with a diameter of 6 mm were punched out of the Whatman cards and then introduced into a Chromacol HPLC vial (with a 300 µL insert). All subsequent extraction steps were performed in the same vial, allowing analytical samples to be withdrawn from the extract with minimal sample loss. Extraction protocols for different biofluids are described below.

2.2.3.1 Serum IVDE

Figure 2-1 illustrates the use of the IVDE method for the extraction of serum spots. 10 µL water was added to each vial containing a sera spot, followed by 40 µL methanol for protein precipitation. Samples were vortex-mixed for 2 min, after which 200 µL of MTBE was added to each sample. Samples were vortexed at room temperature for 60 min. Following addition of 50 µL water per vial, the samples were centrifuged at 3000 r.c.f. for 10 min. This resulted in a clear separation of MTBE (upper) and aqueous (lower) phases, with protein precipitate aggregated at the bottom of the vial. Upper and lower phases could thus be injected directly into

the LC-MS system in two separate runs, by adjustment of injection needle height (15 mm for upper phase, 5 mm for lower).

2.2.3.2 In-vial extraction (IVE) of urine, CSF and cell nutrient fluid (RPMI)

Water (50µL) was added to each vial containing urine, CSF or cell nutrient fluid spots. Proteins were precipitated with 150 µL methanol and vortex-mixed for 2 min, followed by centrifugation at 3000 r.c.f. for 10 min. The sample was then injected into the LC-MS system directly from the vial.

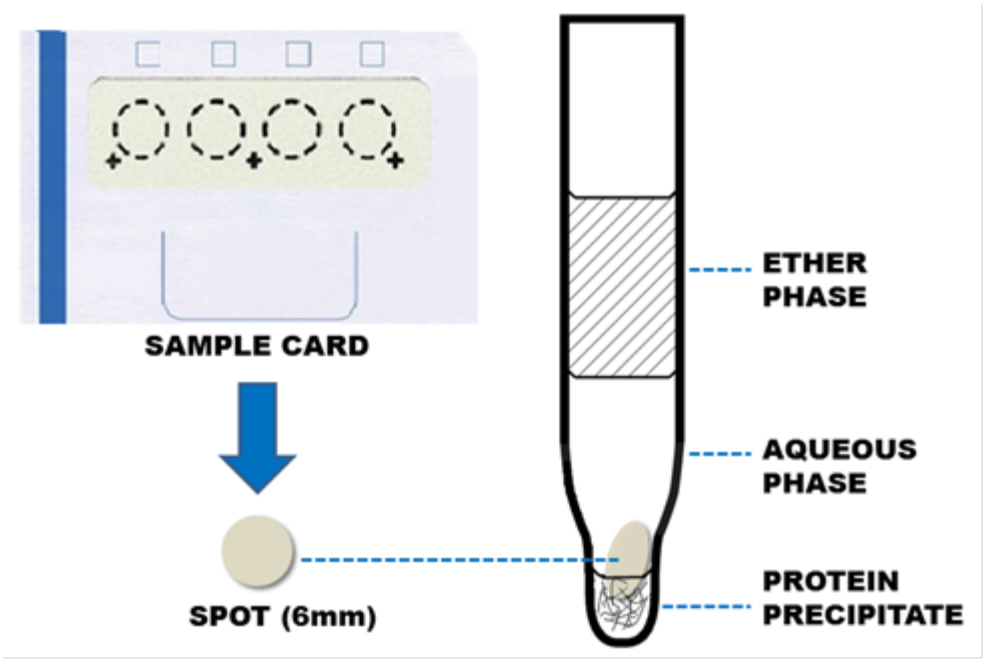

Figure 2-1 In-vial dual extraction (IVDE) of serum spot samples. Top left is a Whatman FTA DMPK-C sample card, with each circled area bearing 10-15 µL of biofluid. A 6mm (diameter) spot (bottom left) is punched out from the card and inserted into a Chromacol vial with a 300 µL glass insert (right). Extraction solvents are added in the manner described in the Methods section, and the resultant solvent bilayer serves for non-targeted general extraction of polar and non-polar analytes.

2.2.4 Analytical Set-up

LC-MS analysis was completed using a Waters ACQUITY UPLC® coupled to a Waters Xevo® G1 QTOF (Waters: Milford, USA). Sample extract (5 µL) was injected onto a reversed-phase C18 column (Supelco Discovery HS C18, 150 mm × 2.1 mm i.d., 3 µm particle size). Separation was performed at 25 °C with a flow-rate of 0.2 mL/min, solvent A composed of 0.1% formic acid in water, and solvent B composed of 0.1% formic acid in methanol. Metabolites were eluted using the following gradient elution method: from 0% B at 0 min to 100% B in 10 min, returning to initial conditions in 1 min and holding for 4 min. Blank extractions were inserted in every 10 samples throughout the LC-MS run to avoid any carry-over effect. A feature is any *m/z* signal maximising at an individual retention time. **Figure 2-2** shows an example of a chromatogram from rat urine that clearly illustrates the density of molecular features obtained using this gradient elution method.

MS analysis was completed in separate positive and negative mode experiments. In both modes, all analyses were acquired in LockSpray mode to ensure accuracy and reproducibility; leucine-enkephalin was used as a lock mass (*m/z* 556.2771) at a concentration of 500 ng/mL and a flow rate of 10 µL/min. Data was collected in the centroid mode over the *m/z* range 100–1000 with an acquisition time of 0.1 seconds a scan. In positive ion mode, the XEVO QTOF was operated with a capillary voltage of 3.2 kV and a cone voltage of 35 V. The extraction cone was set to 2.0 V. The desolvation gas flow was 400 L/hour and maintained at 400 °C. The source temperature was 120 °C and cone gas was set to 50 L/hour. In negative ionization mode, the XEVO QTOF was operated with a capillary voltage of -2.6 kV and a cone voltage of 45 V. The extraction cone was set to 2.0 V. The desolvation gas

flow was 800 L/hour and maintained at 350 °C. The source temperature was 120 °C.

All data was collected using Waters MS^e technology, at two constantly interchanging energies. This enables data collection at two "levels". Level one is traditional MS raw data with parent molecular ionisation. Level two initiates high collision energies within the MS source, which has the effect of causing molecular fragmentation, meaning accurate mass fragmentation data can be collected simultaneously with accurate mass precursor ion data. In this instance, data was acquired at level one with a low collision energy of 5 V, whereas level two acquired the data at collision energy of 50 V.

2.2.5 Data processing and analysis

All data was processed within the Masslynx application manager 4.1 (Waters: Milford, USA), and exported into SIMCA version 13 (MKS Umetrics AB, Sweden) for multivariate analysis. Data files collected in MassLynx were imported into MarkerLynx (a module within MassLynx) for data pre-treatment, including peak alignment and normalization to total peak area (per chromatogram). Processed data was subsequently filtered using an in-house algorithm, which allowed the selection of normalized features present in > 50% of samples within each biofluid group (e.g. within the rat CSF group, within the mouse urine group, etc.). Filtered data was then pareto-scaled and subjected to principal component analysis (PCA) in SIMCA. Note that data is mean-centered as a matter of course during PCA modelling in SIMCA software. Graphical outputs were prepared in SIMCA, as well as in the XCMS (Smith, Want *et al.* 2006, Tautenhahn, Bottcher *et al.* 2008,

Benton, Want *et al.* 2010) and VennDiagram (Chen and Boutros 2011) packages in R (R-Core-Team 2013).

2.3 Results and discussion

The use of dried biofluid spots for the acquisition of semi-quantitative metabolomics data is advantageous for a number of reasons. Firstly, there are the practical benefits associated with easier storage and safer transport alternatives available to spot samples. Secondly, there are important ethical and experimental reasons for pursuing the use of viable small-volume sample spot methods. Burnett (Burnett 2011) summarises the ethical benefits of spot analysis, which include a reduction in the number of animals or participants needed (such as the use of single animals for TK studies), as well as method refinement via the use of less invasive collection procedures. The latter is possible as 10-20 μL samples can be acquired using anticoagulant-coated glass capillaries, which is infeasible for conventionally collected 0.3-0.5 mL blood volumes. It is worth noting that the long-term stability of components in spots has not been thoroughly investigated, even though spot freezing upon acquisition has been recommended for metabolomics applications.(Michopoulos, Theodoridis *et al.* 2011) However, the aforementioned practical and ethical advantages associated with spot analysis, when combined with the concomitant decrease in man-hours needed for sample collection and processing, could lead to broad adoption of the spot methodology in the near future.

Here, we applied the in-vial extraction protocol to the analysis of dried biofluid spots. The combination of small volume spots format with the low loss, high

coverage IVDE method yields reproducible results in a validation set of samples, with low inter-sample variability.

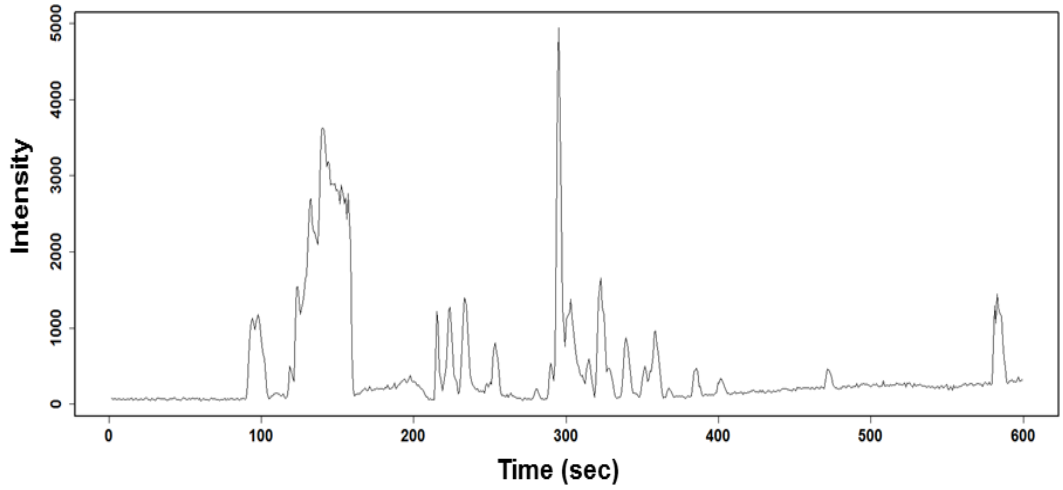

Figure 2-2 Example base peak intensity ion chromatogram of mouse urine extract (positive ionization mode).

2.3.1 Spot-IVDE reproducibility study

To test the reproducibility of the extraction and analysis protocols when applied to the spot samples, 10 spots bearing 10 µL rat serum and 10 spots bearing 10 µL cell nutrient medium were studied. Spots that were 6 mm in diameter were punched out of the Whatman cards and extracted using either a modification of the previously published IVDE method (Whiley, Godzien *et al.* 2012) (rat serum) or a methanol: water extraction solvent (RPMI cell nutrient medium). The combination of aqueous and organic phases in the IVDE method permits the extraction of both polar and non-polar metabolites without transfer-associated sample loss.

The 10 replicates from each method were analysed using identical LC-MS protocols and fingerprints with > 4000 distinct features per chromatogram were obtained. After data processing for noise reduction and total normalization, a set of

six features from each type of extract (i.e. organic and aqueous extracts for serum, aqueous extract for RMPI), representing a range of retention times and masses, were chosen for the analysis of reproducibility. In order to select peaks for reproducibility analysis, the raw data was inspected for *m/z*-retention time features that were present in every sample of the groups in **Table 2-1**. The raw data was then processed using the Waters QuanLynx application's ApexTrack algorithm, which performed mean peak smoothing and baseline correction prior to peak detection and integration for selected masses. Output integrated peak areas were then used for the analysis of variation within the validation groups. Relative standard deviation (RSD) for the peak areas of these metabolites ranged from 1.4-9.4% RSD for these metabolites, an acceptable level of variation (see **Table 2-1**). Processed data from both positive and negative modes was further parsed using an unsupervised multivariate analysis (MVA) principal component analysis (PCA) model. A PCA model best explains the variability in the data, and to quantify this variability, the distance between the two principal vectors was measured for the different types of extract. This vector distance was obtained from the difference between the maximum and minimum vector scores for each group of replicates. In **Figure 2-3**, the PCA scores can be observed for the data collected in positive ionization mode. The variability within the swarm of replicates for each group (calculated as the maximum and minimum vector distance in both PC1 and PC2 directions) corresponds to the serum ether extracts (averaged RSD of 15.1%), followed by aqueous serum extracts (8.1%) and RPMI extracts (5.7%) respectively. The PCA analysis shows that the cumulative fingerprint of the ether group was less reproducible (i.e. showed more spread) than the other sample extracts. However the univariate variation (as measured by the % RSD) for the six analytes chosen for the

serum ether extracts was similar to that of the metabolites from the other extracts (see **Table 2-2**). This higher variability in the lipophilic extracts and fingerprint could be attributed to increased peak overlapping at the higher organic gradient conditions. In our previous work injection of the ether layer was highly reproducible for single features, showing 821 with variation RSD < 5 % when triplicate extractions were performed (Whiley, Godzien *et al.* 2012).

Table 2-1 Peak area variation for six selected analytes from each extract type in the test set.

(a) serum-upper layer				
M/Z	RT	Average Peak Area	RSD(%)	RSD(%) for ratio[a]
178.12	5.11	458.79	5.48	3.70
647.25	**5.87**	48.69	5.98	-
491.18	5.92	52.45	3.83	3.36
205.04	6.18	887.27	9.41	9.97
674.51	11.93	449.04	6.61	7.26
765.49	12.23	527.25	6.96	7.78
(b) serum-lower layer				
M/Z	RT	Average Peak Area	RSD(%)	RSD(%) for ratio[a]
378.90	1.79	224.35	1.39	3.38
182.08	**5.82**	1271.42	3.50	-
250.04	7.01	117.34	3.59	3.81
188.07	7.79	6860.07	4.46	1.83
801.03	9.73	130.01	2.50	3.13
550.63	12.66	66.56	6.44	6.83
(c) RPMI1640				
M/Z	RT	Average Peak Area	RSD(%)	RSD(%) for ratio[a]
242.93	1.75	304.85	4.74	7.20
497.06	2.05	62.80	3.47	5.31
188.07	**8.04**	294.01	3.59	-
349.18	8.34	30.56	5.92	5.86
171.10	9.16	1179.99	8.90	8.52
413.27	14.58	3264.73	5.18	6.89

[a] calculated ratio = Peak Area of feature/ peak area of bolded features in a, b and c (chosen with medium % RSD). This allows for comparison in each group in a normalised fashion.

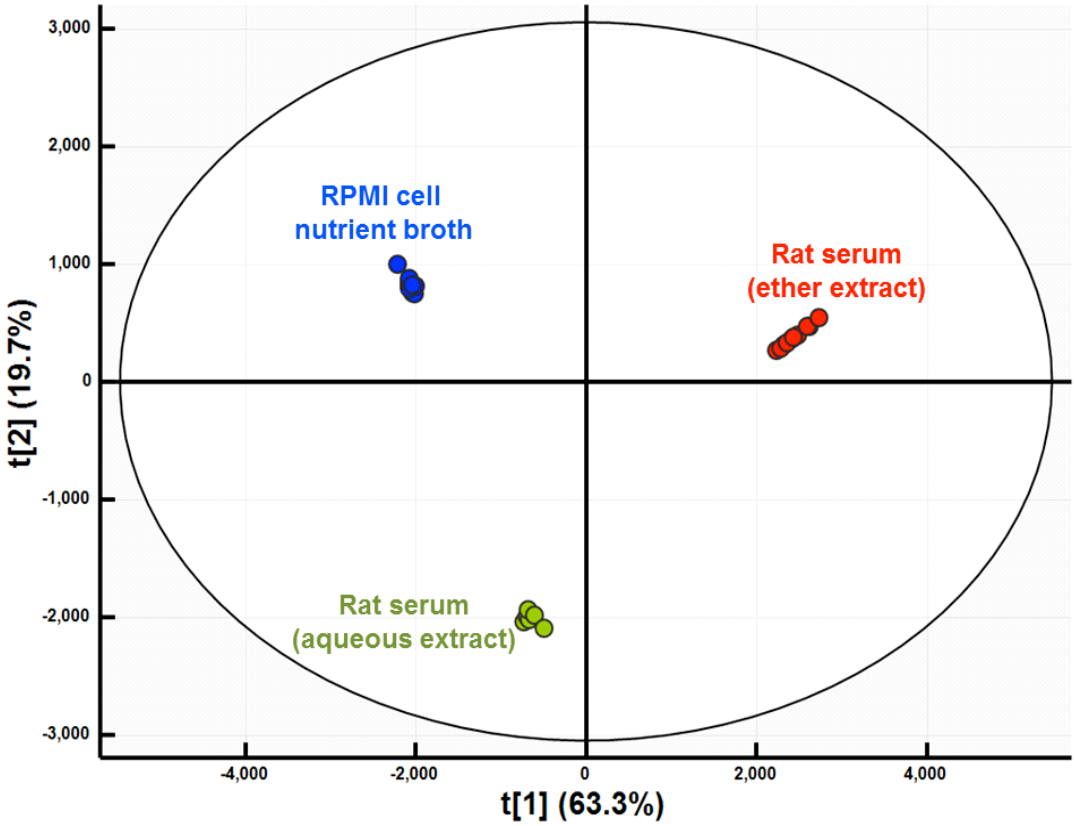

Figure 2-3 PCA plot depicting the variation of spots for rat serum (both organic and aqueous extracts) and for the aqueous (methanolic) extract of the RPMI cell nutrient fluid.

Table 2-2 Variation for each extract type in the test(validation) set as calculated from the PCA scores plot, where variation is calculated per group of 10 replicates as dispersion observed for the two first PC vector scores (t[1] and t[2]).

	(a) serum-upper layer		(b) serum-lower layer		(c) RPMI1640	
	M1.t[1]	M1.t[2]	M1.t[1]	M1.t[2]	M1.t[1]	M1.t[2]
Mean scores value	2450.87	379.16	-642.67	-2015.97	-2065.26	830.42
Δ*	504.69	284.91	241.63	160.24	211.39	255.70
RSD(%)	6.55	23.75	13.60	2.70	2.85	8.55

*Δ = max. vector score – min. vector score (for each group of replicates, per component)

Since small changes in metabolite concentrations can indicate important abnormalities in metabolic pathways, analytically robust methods for metabolomics fingerprinting are essential in order to extract meaningful biomarker data. However analytical variation of some magnitude will always be a factor when assessing potential markers in metabolic fingerprint data. LC-MS as a tool for quantitative analysis can generally show inaccuracies of 10% to 15% RSD, depending on factors such as the type of molecule being detected, absolute concentrations, and matrix effects (Ellison SLR 2012), and this variability depends upon the relationship between these factors and molecular ionization behaviours (e.g. ion suppression or enhancement phenomena) (Xu, Ji *et al.* 2013). In recent studies with UHPLC-MS/MS (Fortuna, Ragazzoni *et al.* 2013), 13% RSD variation was accounted for in a conventional drug pharmacokinetic assay in plasma. While a small sample analysis where sensitivity is compromised, such as in hair analysis, variation of 11.5% was recorded in another validated method.(Roth, Moosmann *et al.* 2013) In addition, the recoveries of tacrolimus from blood spots can have a wider 135% allowed bracket in validation studies.(Li, Cao *et al.* 2013)

2.3.2 Inter-animal and intra-animal fingerprinting comparison

As a proof of concept the method described above was applied to spots bearing three different biofluids (CSF, urine and serum) from healthy rats and mice. After a systematic process of data cut-off and mining which consisted of recording features that were present in at least 50% of the group (per biofluid and per animal), a total of 1182 (positive ionization) and 2625 (negative) features were recorded.

Figure 2-4 illustrates the overlap in molecular features acquired per animal and per biofluid in the form of Venn diagrams. Feature selection was performed on data processed in the Waters MarkerLynx application, using a mass tolerance of 0.05 Da and a retention time window of 0.2 min as threshold values for peak detection and grouping. There is no carry-over found in blanks and they were excluded prior to the feature detection. Features were considered matched if they had the same *m/z* and retention time values after processing, and the Venn Diagrams were produced based on these matched features. From **Figure 2-4 B, C, E and F**, it is evident that the greatest overlap was seen for metabolites from urine and CSF.

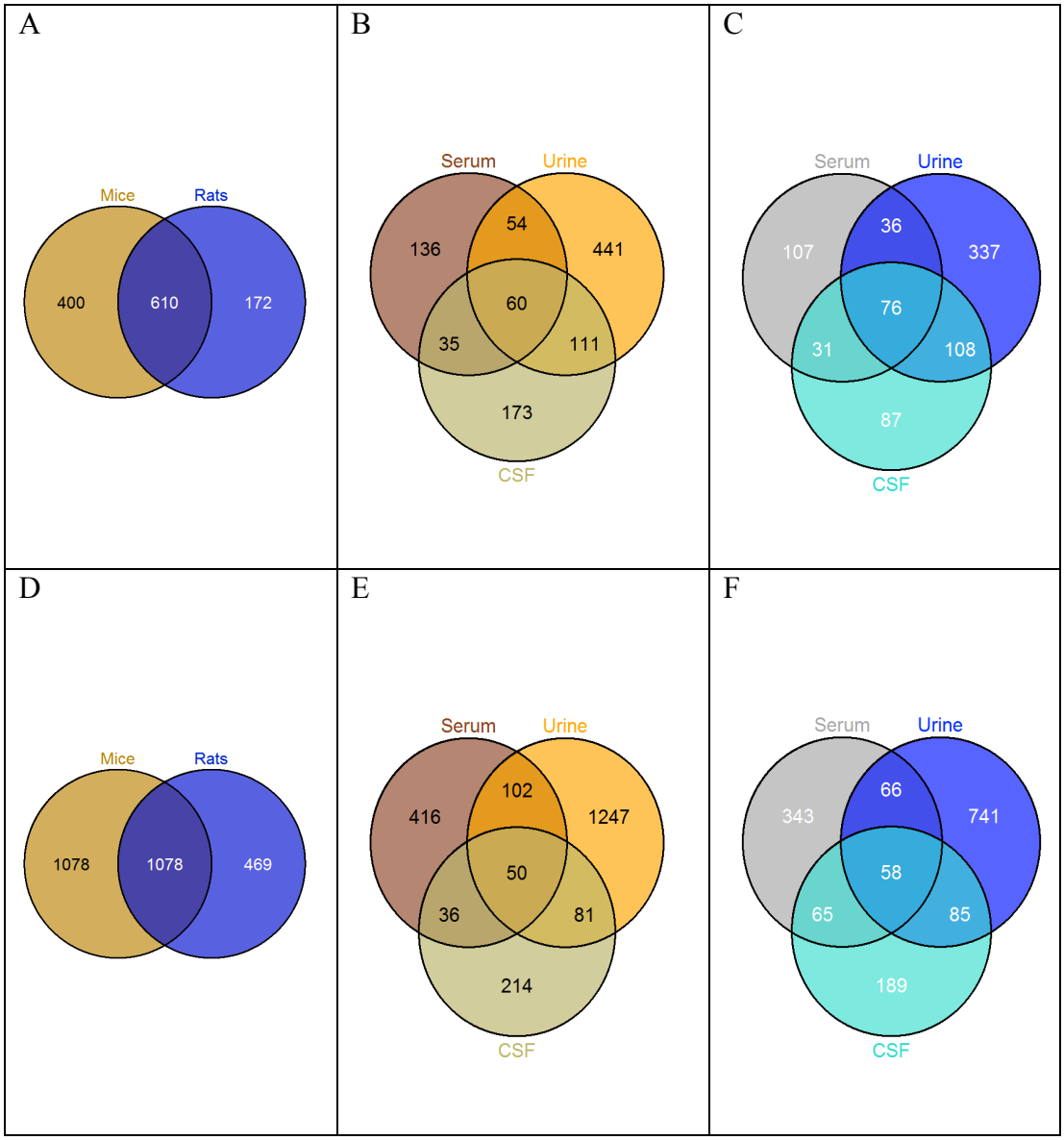

Figure 2-4 Venn diagrams depicting the distribution of molecular features extracted from rodent biofluids. A total of 1,182 and 2,625 distinct features were observed. A, Overlap between features found in mice and rats (positive ionization mode). B, Overlap between features found in all three murine biofluids (positive mode). C, Overlap between features found in all three rat biofluids (positive mode). D, Overlap between features found in mice and rats (negative mode). E, Overlap between features found in the three murine biofluids (negative mode). F, Overlap between features found in the three rat biofluids (negative mode). Figures were prepared using the VennDiagram package (Chen and Boutros 2011) in R (Smith, Want et al. 2006, Tautenhahn, Bottcher et al. 2008, Benton, Want et al. 2010).

Numbers of features in serum were decreased when compared to previous work in our lab where +1500 features were reproducibly acquired from 20 µL of plasma

(Whiley, Godzien *et al.* 2012). It must be noted that the 15 min gradient used for spot analysis in this study is much shorter than the 60 min gradient used for the previous analysis of plasma by IVDE. This reduction in gradient length is a factor worth considering since the number of distinguishable features diminishes greatly as the run length decreases. Such a diminishment can mean an inability to detect small changes in metabolite profiles, such as those associated with lower dose treatments or with changes in low-concentration metabolites. For spots to be a viable alternative for metabolomics studies in animal models, extensive additional work with validation steps will need to cover all aspects of sample handling, shipment, storage, processing (e.g. biofluid temperature and viscosity, punch size, etc.) which have not been considered in the experimental procedure presented here. However, the presented combination of faster screening methods with low sample volumes is likely to be preferred when hundreds or thousands of samples are being investigated in a single experimental design.

2.4 Conclusions

An analytical method for dried biofluid spot metabolomics was designed and tested using samples from rat serum and RPMI cell nutrient medium. 10 µL serum and 10 µL cell medium spot samples were extracted using in-vial extraction protocols, and selected metabolites of varying lipophilicity and mass were chosen for evaluation of method reproducibility. Peak area variations for these selected metabolites were found to be 1.4-9.4% RSD, an acceptable range for conventional LC-MS procedures. When cumulative fingerprints were subjected to standard multivariate statistical data treatment, fingerprints from the serum ether extracts had the highest variability (15.1 % variation), while the serum aqueous extracts and RPMI cell

nutrient liquid aqueous extracts accounted for 8.1% and 5.7% variation respectively. To test the utility of the method in an animal model, the spot-IVDE method was used in the analysis of 86 spots of three different biofluids, which were obtained from healthy rats and mice. A total of 1182 features were recorded in positive ionization mode and 2625 in negative and from those, 610 and 1078 respectively were found to be shared in mice and rats. Furthermore, a higher overlap of molecular features was observed between urine and CSF spots for both animals. With this work we have clearly shown that the application of the IVDE method to dried biofluid spots results in reproducible metabolic fingerprints, with sufficient feature coverage for high-throughput metabolomics experiments. The small sample sizes (10-15 μL) required for the spots, and the low transfer losses associated with the IVDE method, suggest that the spot-IVDE procedure should be widely adopted, in particular for large-scale metabolomic studies.

Paper spots will be compared with the conventional sampling method and further assessed for storage stability in Chapter 4, utilising the analytical and data-treatment protocol designed in Chapter 3. The protocol will be tailored for high resolution and high mass accuracy instruments in order to aid with the identification of molecules, and can be applied to the discovery of doping biomarkers in later chapters.

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Chapter 3 Development of an analytical and data-treatment protocol for metabolomics using a UHPLC-Q-Exactive™ Orbitrap-MS

3.1 Introduction

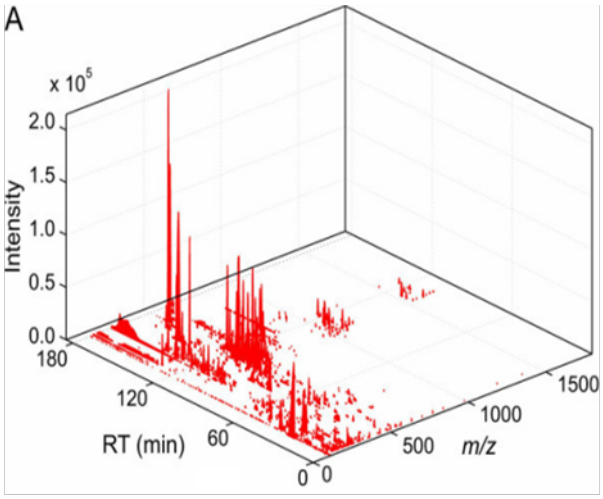
An entire metabolomics experimental workflow includes sample preparation, instrumental analysis, data treatment and biological interpretation. Among these, instrumental analysis and the following data treatment are considered crucial steps, as metabolomics is highly dependent on robust methodology. Various techniques can be applied in metabolomics and protocols that give increased metabolic information while maintaining the cost of the analysis are preferred. It is beneficial for a metabolomics research laboratory to know how to make the best use of the analytical instrument and to develop a standard instrumental and data-treatment protocol.

In sample analysis, liquid chromatography (LC) combined with mass spectrometry (MS) is the most used instrument for metabolomics showing high metabolic coverage (Want, Wilson *et al.* 2010). High mass resolution is particularly important for all types of experiments involving complex mixtures (e.g. biological, environmental, food), since these contain a significant number of background (matrix) ions in addition to the possible analytes of interest. Metabolomics usually focuses on such complex samples, thus, high-resolution accurate-mass (HRAM) mass spectrometry is more and more preferred in metabolomics field due to its capacities of analysis of unknowns, confirmation of analyte structure, retrospective search for new compounds and addressing sensitivity requirements.

However, a challenge associated with metabolomics, as well as other "omics" studies is that huge and complex datasets are generated and those need to be simplified. In LC-MS based metabolomics, the raw data are in complex 3-D

combination format of chromatogram and spectrum, and data pre-processing need to be first applied to reduce the dimension of the data to 2-D format (**Figure 3-1**). Markerlynx is a software provided by Waters Corporation and specifically processes the fingerprinting data from Waters Quadrupole Time-of-flight Mass Spectrometry (QTOF-MS). Our laboratory has previously developed a metabolomics workflow using Markerlynx (Whiley, Sen *et al.* 2014), and Chapter 2 was based on the established data pre-processing procedures. However, Markerlynx works only for Waters instruments and processes relatively small data sets (e.g. up to 200 samples), thus new data pre-processing techniques are urgently needed. XCMS has emerged as a powerful metabolomics data processing method. It is available from the free-access programming software R, and highly adaptable to almost all types of LC-MS and GC-MS data sets with the help of format conversion tools (Smith, Want *et al.* 2006, Tautenhahn, Bottcher *et al.* 2008).

Even after pre-processing, datasets still comprise large number of metabolic features (1K-40K) that are difficult to summarize and visualize. Multivariate analysis is usually employed for the analysis and modelling of complex data. Models can be non-supervised chemometrics method, e.g. principal component analysis (PCA), where outliers and overall data distribution will be assessed, or supervised, such as partial least-squares (PLS), partial least-squares discriminate analysis (PLS-DA) and orthogonal partial least-squares discriminate analysis (OPLS-DA), where grouping of samples is possible to select their descriptive metabolic features or variables (Wiklund, Johansson *et al.* 2008).



	A	B	C	D	E
1		N01P-2	N02P-2	N03P-2	N04P-2
2	M100.0244T90.80	70291214.46	42615622.5	33826471.37	32586819.85
3	M100.0757T259.25	100354891.3	45988636.56	100225446.3	116710329.2
4	M100.1121T356.16	78910970.44	56777294.51	31114515.89	40088066.99
5	M100.9319T77.76	16791091.39	5715549.364	15674858.65	44126760.31
6	M100.9318T11.75	9401451.151	2574575.747	16228336.54	11328987.4
7	M101.0197T113.09	30332141.24	4123980.186	9258110.019	9347527.316
8	M101.0197T90.77	9339690.516	91608832.97	131847960.3	123885107.8
9	M102.0205T90.74	9240650.726	11652329.84	15172887.62	15013274.58
10	M102.9703T91.98	50064164.93	14124135.48	56853337.39	155918975.4
11	M103.0390T90.64	50232630.35	12632123.58	18071938.74	18825505.23
12	M103.0543T390.31	29937181.54	3673270.686	160052221.1	3160042.247
13	M104.0706T123.05	136052337	44198805.34	59121703.81	68985563.04

Figure 3-1 Schematic diagram of dimension reduction of LC-MS profiling through data pre-processing. A, visualized combination of LC-MS chromatogram and spectrum. B, an example output table after data pre-processing. At each retention time there are various ions detected. Data pre-processing will give an output of peak area (or intensity) of distinct features with different Mass-to-Charge Ratio (*m/z*) and retention time (RT) in each sample.

The aim of this chapter is to give an optimised methodology to best practice the general hypothesis of this thesis, by developing a metabolomic protocol for the laboratory using UHPLC-HRMS (Q-ExactiveTM Orbitrap-MS). Firstly the analysis conditions will be optimized and various resolutions will be tested for metabolic profiling. The addition of quality control samples can aid the accuracy assessment of the protocol. The data processing protocol includes several steps,

namely, data pre-processing in XCMS, normalization and multivariate analysis in SIMCA. The ability of dealing with complex large matrix and stability of this protocol will then be tested by profiling 101 urine and QC samples. This workflow developed for UHPLC-Q-Exactive™ Orbitrap-MS is shown in **Figure 3-2**:

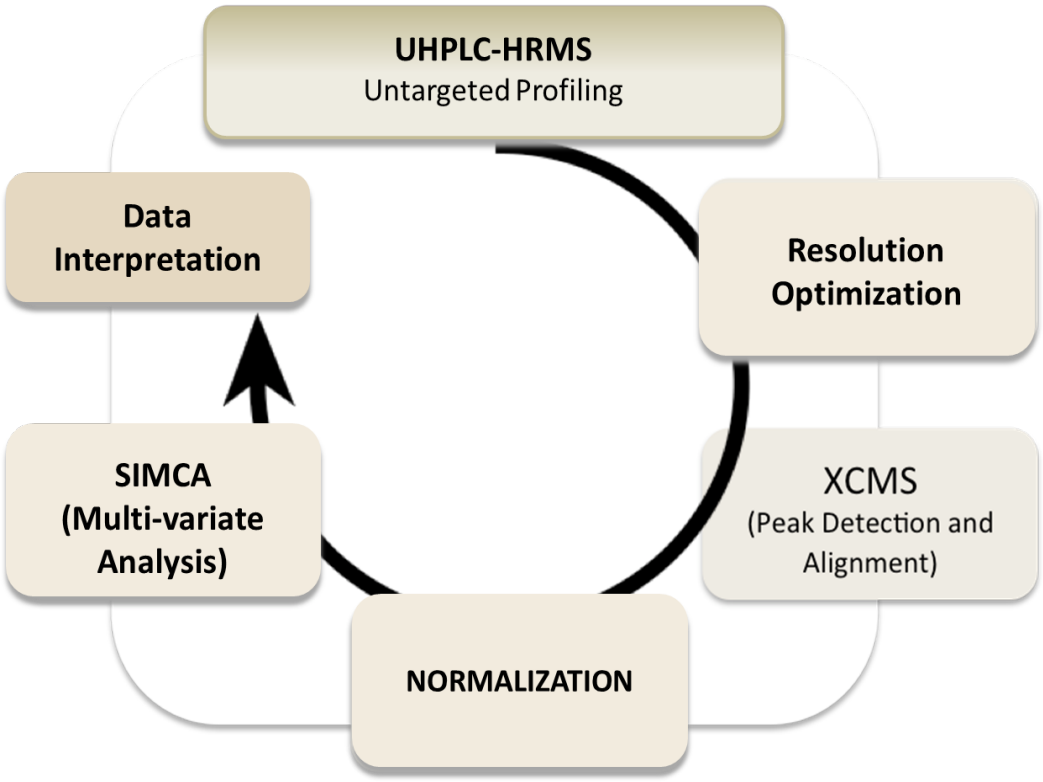


Figure 3-2 General steps of proposed metabolomics data treatment workflow for ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS).

3.2 Methods

3.2.1 Chemicals and materials

All solvents used were HPLC grade. Acetonitrile and methanol were obtained from Fisher Scientific (Loughborough, UK). Formic acid (99–100 %) was purchased from VWR (Leicestershire, UK). Ultrapure water (18 MΩ · cm) was obtained from an ultra-pure water system (Elga, UK).

3.2.2 Urine samples

The samples from anti-doping tests were acquired after professional sport competition and came from diverse demographics at random times of the day. A total of 91 urine samples were obtained from 91 different athletes, who had indicated their consent for research on their samples. For resolution suitability test, two experiments were carried out: 1) One single salbutamol detectable sample (DET) was used and repeated injection for 3 times in different resolutions. 2) Three DET samples and two salbutamol non-detectable samples (NON-DET) were randomly chosen and analysed under different resolutions in MS. A pooled sample from 20 urine samples was used as a quality control (QC). For intra-batch reproducibility test, all the 91 samples were injected into LC-MS and quality controls (QC) were inserted after every 10 samples throughout the whole run. The specific gravity was measured by a calibrated DM40 densitometer (Mettler-Toledo International, Switzerland). All urine samples were aliquoted and stored in polypropylene tubes at -20 °C for at least three months and anonymized prior to analysis to comply with the ethical requirements of the World Anti-Doping Agency.

3.2.3 Sample preparation

All the urine samples were centrifuged at 6,000 r.c.f for 15 minutes to remove any particles. An aliquot of 50 µL of supernatant was diluted with 200 µL ultrapure water. The solution obtained was pipetted into amber HPLC vials with inserts. Then a volume of 10 µL sample in each vial was injected into LC-MS system.

3.2.4 UHPLC–HRMS analysis

For chromatographic separation, a Dionex™ Ultimate® 3000 UHPLC system (Dionex™, Sunnyvale, USA) was used. Separation was carried out on a reversed-phase C18 column (Supelco Discovery HS C18, 150 mm × 2.1 mm i.d., 3 µm particle size). The column was thermostatted at 25 °C and a flow-rate of 0.2 mL/min was used. Solvent A was composed of 0.3 % formic acid in water, and solvent B composed of 0.3 % formic acid in acetonitrile. Metabolites were eluted using the following gradient elution method: from 0 % B at 0 min to 100 % B in 10 min, returning to initial conditions in 0.5 min and held for 2 min.

Detection was performed by means of a Q-Exactive™ Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with a heated electrospray (HESI-II) ion source, operated in polarity switching mode. The instrument was calibrated before each batch of samples. Source conditions were as follows: sheath gas flow rate, auxiliary gas flow rate and sweep gas flow rate: 70, 10 and 0 arbitrary units respectively; capillary temperature: 320 °C; heater temperature: 350 °C; spray voltage: +3.75 kV (positive ionization mode) and −3.00 kV (negative ionization mode); lock mass: off. Nitrogen was used as both the source and the collision (HCD) gas. Data acquisition was performed at resolutions

of 17,500, 35,000, 70,000 and 140,000 FWHM at *m/z* 200. Each acquisition cycle included a full-scan acquisition in positive ionization mode and a full-scan acquisition in negative ionization mode (polarity switching mode). The AGC target was set to $1e^6$ and the scan range was *m/z* 100–1000 for all acquisition events.

3.2.5 Data processing and analysis

All raw data were collected using Thermo Xcalibur software, before being processed within XCMS in R and XCMS online (https://xcmsonline.scripps.edu) and exported into SIMCA version 13 (MKS Umetrics AB, Sweden) for multivariate analysis. Data files (.RAW) collected in Xcalibur were converted by Proteomic Wizard (http://proteowizard.sourceforge.net/) to .mzXML format, before importing into freely available XCMS package in R and XCMS online. For peak picking the following parameters were used: method = 'centWave', ppm = 4, peakwidth = c (6, 30), prefilter = c (10, 5000). For peak grouping, bw = 5, mzwid = 0.05 were used. After peak alignment to allow minor retention time variation and filling missing peaks, an output table of time-aligned detected features was exported with their retention time, *m/z* ratio, and intensity in each sample. A feature is any *m/z* signal maximizing at a specific retention time. The resulting tables were readily exported to Microsoft Excel and feature numbers were obtained in the Excel output tables. Then the data collected were normalized by the specific gravity (SG) of each sample and ready for multivariate analysis. Levine-Fahy equation was used for SG-normalization:

Concentration SG normalized = Concentration specimen • (SG reference - 1)/ (SG specimen – 1) (Cone, Caplan *et al.* 2009)

SG reference is a population reference value for specific gravity representing "normal" or "non-diluted urine" and SG specimen is the specific gravity of the test urine specimen. The SG reference utilized in all corrections was 1.0200 (WADA 2014).

3.2.6 Statistical analysis and data interpretation

The data from both positive and negative ionization mode were then normalized by the specific gravity of each sample, before importing into SIMCA (UMetrics, Umeå, Sweden), where they were subjected to multivariate data modelling, including pareto-scaled principal component analysis (PCA). The cloud plots were prepared in XCMS online. For intra-batch reproducibility test, QCs were inspected for clustering in PCA and a technical variation smaller than 15% of total inter-sample variation (calculated as the maximum and minimum vector distance in both t[1] and t[2] directions) was deemed acceptable for metabolomics data.

3.3 Results and discussion

3.3.1 HRMS resolution selection

Resolution selection is a crucial step for untargeted metabolomics analysis. High-Resolution Mass Spectrometry (HRMS) provides accurate mass measurement, for Q-Exactive the resolution can go up to 140,000 FWHM (at *m/z* 200), with a mass accuracy < 1ppm with internal calibration. However as the resolution increases, the scan speed drops, leading to reduced sensitivity. While the maximum scan speed can reach 12 Hz at the resolution setting of 17,500 in single polarity and full mass scan mode, the scan speed at resolution of 140,000 will dramatically drop to

less than 1/10 of that amount. Studies using Orbitrap instruments to analyse complex matrix have chosen different resolution settings: Vanhaecke *et al.* used Fourier Transform (FT) Orbitrap MS at 50,000 FWHM for full MS scan in search for growth-promoting agents in meat (Vanhaecke, Van Meulebroek *et al.* 2013). Xu *et al.* used three Orbitrap MS instruments for targeting of 137 chemical compounds: 30,000 FWHM for the LTQ-Orbitrap Discovery and 100,000 FWHM for the LTQ-Orbitrap XL and the Exactive, which is the top resolution both instruments can reach (Xu, Heilier *et al.* 2010). Raro *et al.* used Q-Exactive Orbitrap MS at 35,000 FWHM to do untargeted metabolomics, however there was no reasons provided for choosing that specific resolution (Raro, Ibanez *et al.* 2015). Thomas *et al.* acquired full scan data at 70,000 FWHM in polarity switching mode for doping control (Thomas, Geyer *et al.* 2012).

Polarity switching mode is a practical feature of the Q-Exactive, which is a positive and negative ionization scan-to-scan alternating mode, and both spectra can be obtained in one injection. Thanks to this feature the analysis time will be cut into half and it is especially helpful when sample volume is limited, which is ideal for large-scale metabolomics analysis. However operating in polarity switching mode will also reduce the scan speed to half. In order to determine the best resolution for untargeted metabolomics and to build a standard protocol for our laboratory, different resolutions of 140,000, 70,000, 35,000, and 17,500 FWHM in polarity switching mode were tested on a urine sample and a pilot study was performed on 5 urine samples in resolutions of 140,000, 70,000 and 17,500 FWHM. The TIC chromatograms in **Figure 3-3** illustrated the good reproducibility of the chromatograms and that the higher resolution used, the less background noise is

observed. **Figure 3-4** demonstrated the selectivity of the 4 different resolutions. It can be observed that an increased mass resolution is associated with a higher mass accuracy and therefore positively affects the selectivity.

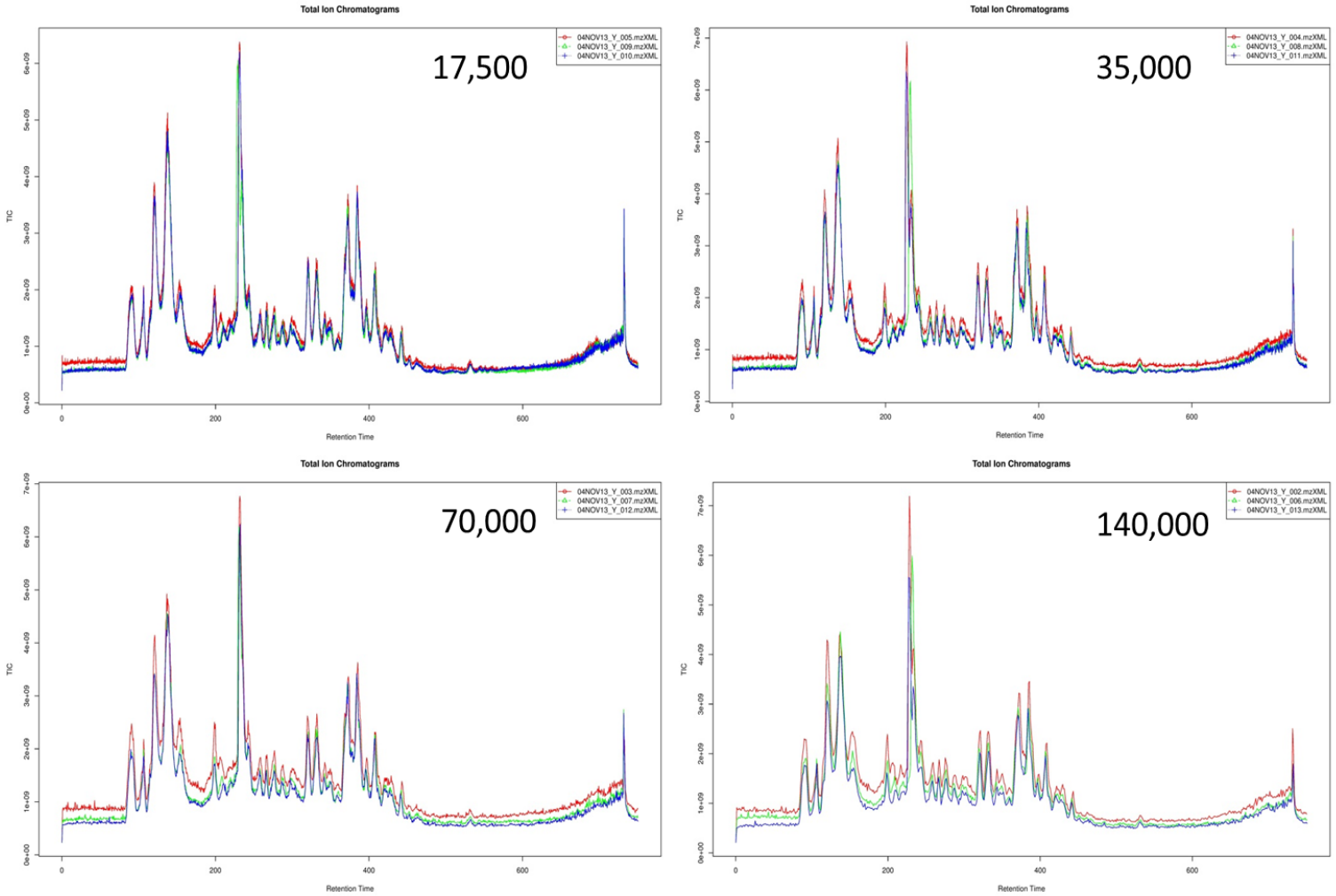


Figure 3-3 TIC of 4 different resolutions of 17,500, 35,000, 70,000 and 140,000 FWHM. There were 3 repeated injections in each resolution tested.

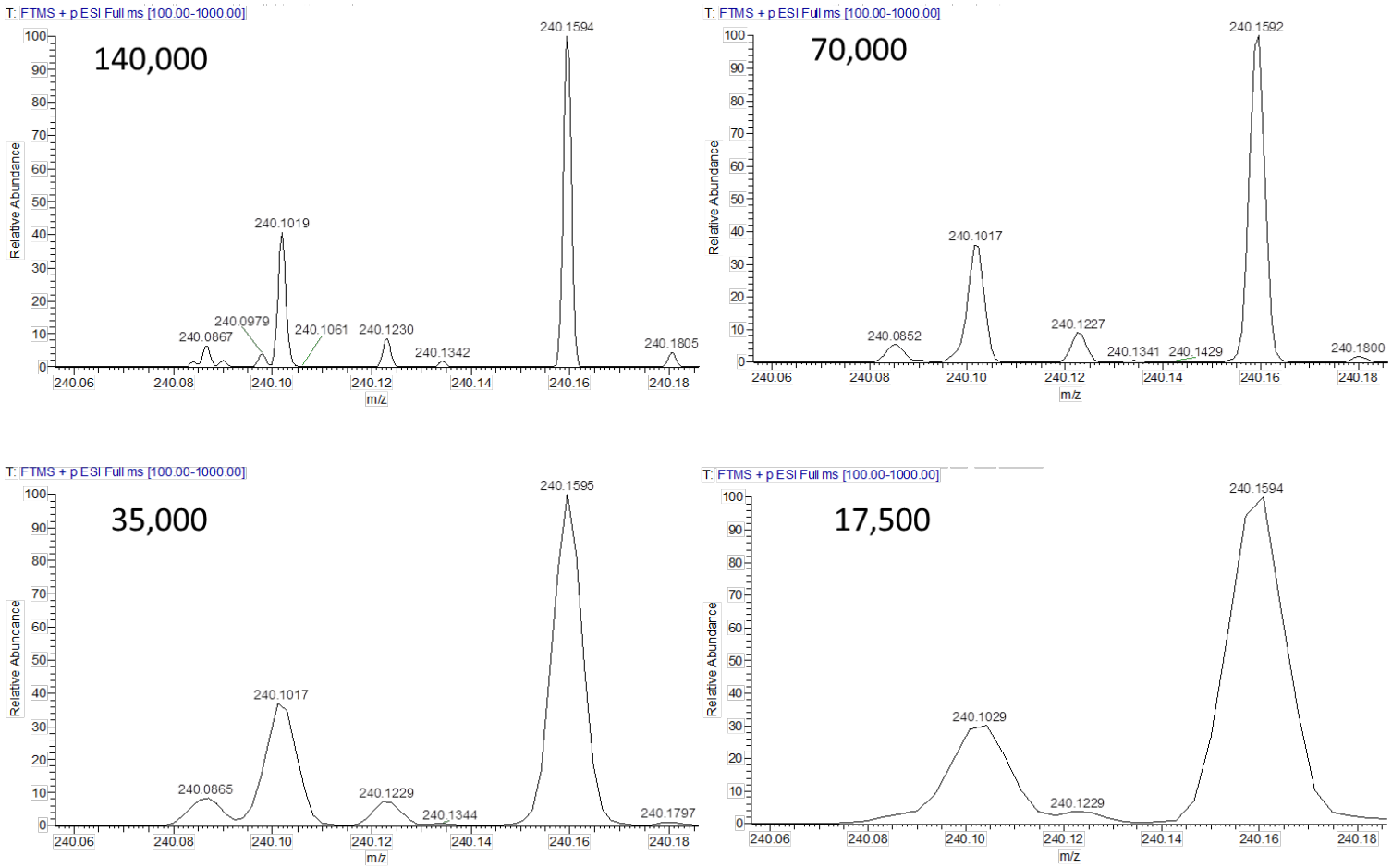


Figure 3-4 Combined mass spectrum during 4.04-4.27 min from m/z range 240.0550 to 240.1850 at 4 different resolutions of 140,000, 70,000, 35,000 and 17,500 FWHM.

However the higher resolution is achieved by longer scan time, and in order to optimize the mass spectrometric resolution for metabolomics, a compromise between selectivity and sensitivity has to be made (Vanhaecke, Van Meulebroek *et al.* 2013). **Figure 3-5** showed the MS spectra of a single molecule, salbutamol, in stick view at different MS resolutions. There were approximately 10, 12, 14 and 14 scans for salbutamol over the 0.2-min retention time range at the resolutions of 140,000, 70,000, 35,000 and 17,500 FWHM, respectively. The calculated corresponding scan rates were 0.62, 0.87, 1.06 and 1.11 Hz. For qualitative analysis, a peak composed of more than 3 scans can be considered valid; however for quantification usually at least 12-15 scans points per peak are needed. In untargeted metabolomics, semi-quantification is normally used and strict quantitation rules are not applied; however the discovery of potential biomarkers relies on the relative concentrations of metabolites in different groups. With fewer scans the accuracy of quantification will drop and furthermore, the slow scan speed could miss some low intensity features that quickly pass the detector. For this work, HRMS was the instrument of choice for our metabolomics study because of the advantages of high mass accuracy, which is essential for identification in untargeted metabolomics. Considering the balance between the selectivity and sensitivity, from this stage, the resolution of 70,000 was considered to be a plausible resolution with a mass error still less than 3ppm.

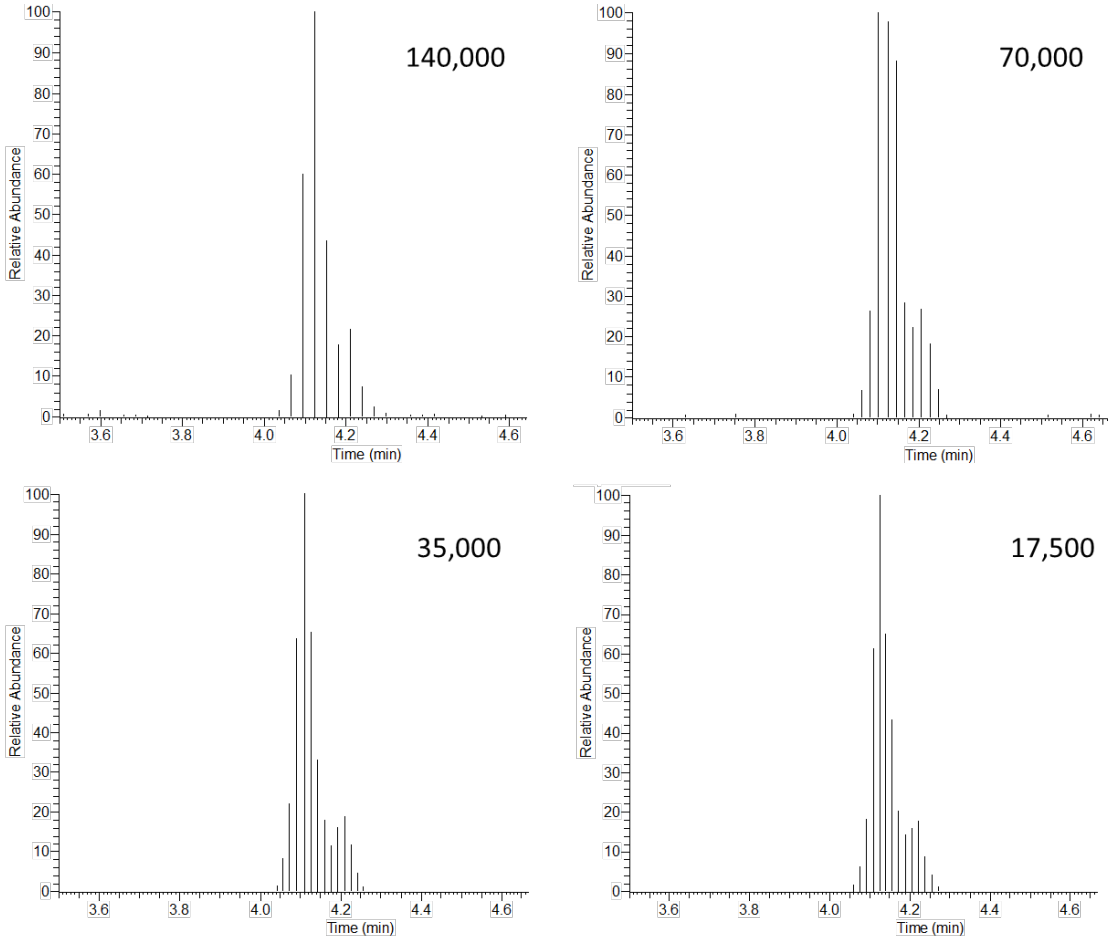


Figure 3-5 MS spectra of salbutamol (in stick view, *m/z* = 240.1594) at different resolutions of 140,000, 70,000, 35,000 and 17,500 FWHM. There were approximately 10, 12, 14 and 14 scans for salbutamol over the 0.2-min retention time range at the resolutions of 140,000, 70,000, 35,000 and 17,500 FWHM, respectively. The calculated corresponding scan rates were 0.62, 0.87, 1.06 and 1.11 Hz.

In the following sections, the data treatment stages would determine if the resolution of 70,000 could provide the amount of information needed for metabolomics.

One possible drawback when comparing this new protocol with the laboratory's established metabolomics protocol was that previous research in our group using QTOF usually applied MS^e fragmentation at the same time during the MS analysis.

However, in this study we found that adding fragmentation would further reduce the scan speed. Thus it was proposed that full mass scan would be used and then fragmentation mode could be run on selected samples. Hence after data analysis, selected features can be fragmented and compared with the pure standards when possible.

3.3.2 Data pre-processing

Before statistical analysis, the raw data obtained from LC-MS need to be subjected to pre-processing to change from 3-D format to 2-D matrix (**Figure 3-1**). This process includes data format converting and XCMS pre-processing. XCMS can only recognise several data formats for processing, such as .CDF, .NetCDF, and .mzXML etc, which are not the raw data format obtained from LC-MS instruments, so an additional conversion is needed. Whilst the raw data generated from Waters Masslynx (.raw) can be converted into .NetCDF format using the Databridge package in Masslynx, the raw files from Thermo Xcalibur (.RAW) can be converted by Proteomic Wizard into .mzXML format.

The converted data then can be processed in XCMS in R or XCMS Online. XCMS is a powerful untargeted metabolomics processing tool and it is operated in programming software R. XCMS Online is an alternative: it is a web-based version of XCMS software that allows users to easily upload and process data without bioinformatic and/or software programming expertise. LC/GC-MS data, and results can be browsed in an interactive, customizable window online showing statistics, chromatograms, and putative METLIN identities for each metabolite (Tautenhahn, Patti *et al.* 2012). The

major drawback of XCMS Online is the time-consuming data uploading, which was not ideal for our research laboratory. In addition, it is also lack of normalization, transformation and scaling steps. Therefore, it was suggested that data pre-processing in XCMS in R would be more practical, followed by normalization in Microsoft Excel, before importing into statistical software such as SIMCA, SPSS or packages in R. This protocol shows multivariate analysis utilising SIMCA which is more user-friendly, to do data transformation, scaling and multivariate analysis.

Several peak-picking, grouping and alignment parameters in XCMS were chosen and tested according to the instrument capacity and settings, raw chromatograms and MS spectrum. Finally, the parameters were set as follows: for peak picking, method='centWave', ppm=4, peakwidth=c (6, 30), prefilter=c (10, 5000) were used; for peak grouping, bw = 5, mzwid = 0.05 were used; for peak alignment, "obiwarp" method was used to correct minor retention time variation. Matrices with time-aligned detected features were exported with their retention time, *m/z* and peak area in each sample. There were 1,946, 3,315 and 1,337 features detected in the resolutions of 140,000, 70,000 and 17,500 FWHM respectively across the 5 test samples, which indicated that the largest amount of information was obtained from 70,000 FWHM.

3.3.3 Normalization method

Since metabolite concentrations across urine samples can vary more than 15 times due to variable water content (Warrack, Hnatyshyn *et al.* 2009), a good normalization method is needed. Some authors use creatinine as the normalization standard (Zheng, Chen *et al.* 2013, Mathe, Patterson *et al.* 2014), but this is not preferred for athletes

since creatinine levels may be affected by protein and creatine intake, as well as catabolic state of the athlete. Other methods such as using internal standard, total peak area, quantile and median-fold change were also described (Kultima, Nilsson *et al.* 2009, Veselkov, Vingara *et al.* 2011, Dai, Yin *et al.* 2014). In doping control, specific gravity is the standard normalization method recommended by World Anti-Doping Agency (WADA) (WADA 2014), hence specific gravity was applied as the normalization method for this urine metabolomic study.

3.3.4 Multivariate analysis

The cloud plots (**Figure 3-6, A-C**) visually showed the discriminant features separating the DET and NON-DET groups, with p-value ≤ 0.01 and fold change ≥ 1.5 in 3 different resolutions. It is observed that there were 96 distinct features in the resolution of 70,000 with p-value ≤ 0.01 and fold change ≥ 1.5, more than the number of 77 in the resolution of 140,000 and 20 in the resolution of 17,500, which further indicated the potential of finding more candidate biomarkers in the resolution of 70,000.

According to the results showed and discussed above, resolution 70,000 was initially chosen for our metabolomic study. To further prove its feasibility, the matrix generated from XCMS in the resolution of 70,000 were imported into SIMCA and subjected to multivariate analysis. The PCA score plot (**Figure3-6, D**) showed the clear separation between DET and NON-DET groups in the resolution of 70,000.

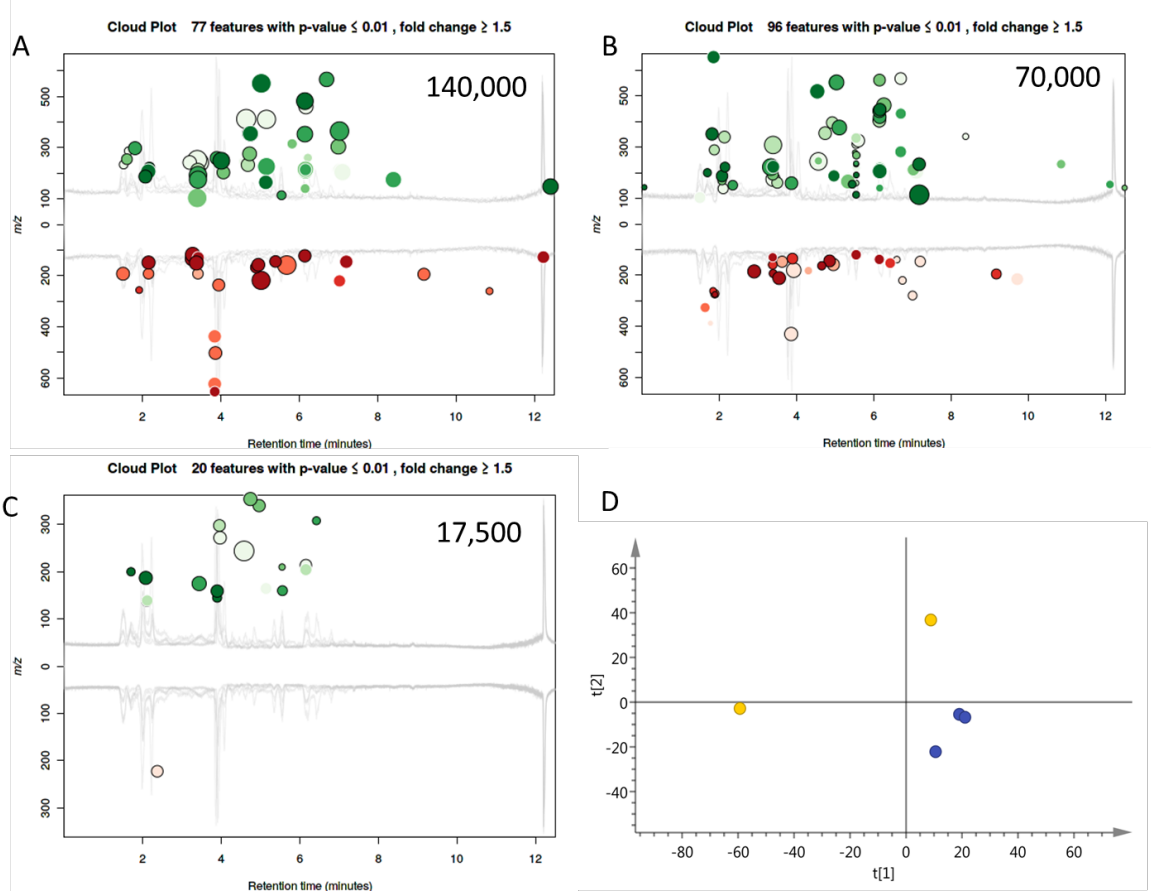


Figure 3-6 Multivariate analysis showing in cloud plots and PCA score plot. A-C, cloud plots of the data from the resolutions of 140000, 70000 and 17500 FWHM, respectively. Green: up-regulated (intensity increased in DET samples) features. Red: down-regulated (intensity decreased in DET samples) features. *p*-value is represented by color intensity (more intense means lower *p*-value). Fold change is represented by the radius of each feature. Retention time is represented by position on the x-axis. Mass-to-Charge Ratio (*m/z*) is represented by position on y-axis. D, PCA score plot of 2 NON-DET (yellow) and 3 DET (blue) samples in the resolution of 70,000.

3.3.5 Reproducibility test on the proposed protocol

To assess the feasibility of the protocol with a bigger number of samples and QC samples, 91 urine samples and 10 QCs were analysed using the proposed protocol with the chosen experimental and data processing parameters. Multivariate analysis results are shown in **Figure 3-7**. PCA score plot is an overview of the entire multivariate data

of observations and their relationships. The "pooled" quality control (QC) urine samples were used to provide a representative mean sample that should be identical or very similar. The QCs clustered together in the PCA score plot, with the distribution measured as 4.4% and 11.9% (at t[1] and t[2] directions, respectively) of the entire sample cohort distribution. Considering the large number of variables in these data (ca. 3,000), the quality of the data corresponding to the method applied was deemed acceptable.

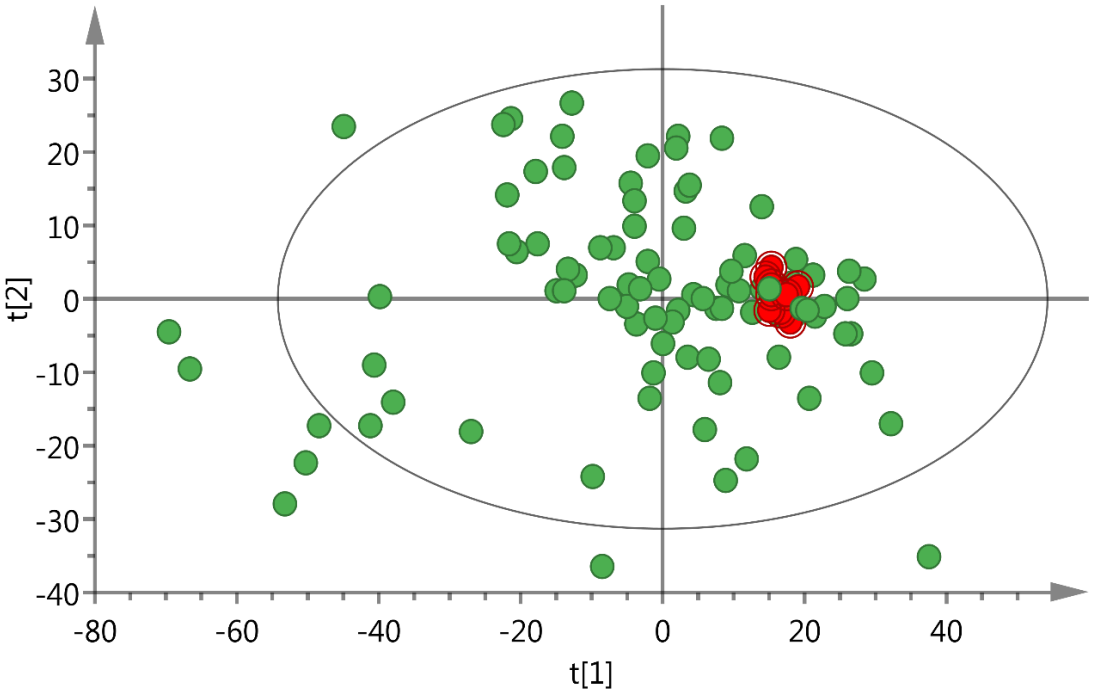


Figure 3-7 PCA score plot of 101 urine samples. Red circles signifies a repeat extraction of a urine pool used as a quality control (QC) (n = 10). The QCs cluster in same area of the PCA, suggesting across-run reproducibility.

3.4 Conclusions

This study developed a protocol for UHPLC-Q-Exactive™ Orbitrap-MS based on non-targeted metabolomics, including instrument resolution optimization and a data mining workflow. Resolutions of 140,000, 70,000, 35,000 and 17,500 FWHM were tested. Two groups (DET and NON-DET) were created according to whether or not containing salbutamol and analysed in the resolutions of 140,000, 70,000 and 17,500 FWHM. For data processing, parameters tailored to high resolution Orbitrap-MS data in XCMS were chosen and specific gravity was used as the normalization method.

The result showed that fingerprinting using this protocol measured more than 3K features in urine at the resolution of 70,000, giving more features compared to the other two resolutions. Multivariate analysis also revealed that the largest quantity of discriminant features (96) was obtained at the resolution of 70,000. Therefore 70,000 was initially chosen as the MS resolution in our protocol and PCA plot showed a clear separation of DET and NON-DET using this protocol.

The protocol was then applied to 91 urine samples and 10 QCs. Multivariate PCA analysis showed overall analytical stability of the protocol across 101 samples, with the distribution of QC samples taking 4.4 % and 11.9 % of the entire sample cohort range at t[1] and t[2], respectively. It is ready for further analysis of data interpretation, including OPLS-DA and S-plot to find the discriminant features, feature annotation and finally biological interpretation.

In the next chapter, this protocol will be combined with the previously developed spots IVE method in Chapter 2, and to compare it with the conventional sampling method as well as the short-term storage stability using urine specimens.

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Chapter 4 Evaluation of dried urine spots using salbutamol as a model non-endogenous metabolite — a metabolomic approach

4.1 Introduction

Chapter 2 demonstrated the potential of in-vial extraction (IVE) of dried biofluid spots as a viable alternative to the use of small volumes of neat biofluids in animal metabolomics studies. In this chapter, IVE of dried urine spot will be further evaluated utilizing the analytical and data-mining protocols developed in Chapter 3.

DBS represent an attractive option for global metabolomics studies as stated in Chapter 1, due to the potential advantages such as using smaller sample volumes, ease of collection, simplified transportation and storage requirements and cheaper overall costs.

Urine is regarded as an ideal biofluid in metabolomics studies, as it captures and concentrates a wide range of metabolites and dynamic processes occurring throughout the body (Emwas, Luchinat *et al.* 2015). Urine generally is prone to changes induced by genetic and environmental challenges reflecting cells and enzyme activity (Walsh, Brennan *et al.* 2006, Walsh, Brennan *et al.* 2007, Fardet, Llorach *et al.* 2008, Scalbert, Brennan *et al.* 2009). Compared with plasma and cerebrospinal fluid, urine is non-invasive to collect and requires less sample pre-treatment as the matrix contains relatively low protein levels.

Sample preparation is considered a limiting step in metabolomics as ideally samples should be kept in their original physiological state. In urine metabolomics, the preferred preparation method is dilution and direct injection of urine into an LC-MS with minimal pre-treatment and preparation to remove particulates (Want, Wilson *et al.*

2010). However, as the inherent complexity of urine containing abundant metabolites with distinct chemical characters and a high dynamic range, sample preparation is a vital step to ensure reproducible, accurate and reliable results in any metabolomics study. More recently, dried paper spots represent a potential sampling method (Deglon, Thomas *et al.* 2012), which as mentioned previously have many apparent advantages over conventional methods such as small volume of samples (typically less than 15 μL), easy sample storage and transport. Paper spots can also lack reproducibility and coverage, but it has been demonstrated in Chapter 2 that the potential of in-vial extraction (IVE) of dried biofluid spots as a viable alternative to the use of small (10−15 μL) volumes of neat biofluids in animal studies to generate reproducible metabolic "fingerprints", with sufficient feature coverage for high-throughput metabolomics experiments (Sen, Wang *et al.* 2013).

The feasibility of IVE dried urine paper spots compared to conventional urine liquid method has not been reported, neither has the comparison conducted in a metabolomics doping test application. Thus one of the aims of this study is to evaluate these two sampling procedures mainly by using untargeted metabolomics, to investigate the IVE paper-spot method in terms of precision and whether or not it leads to information loss.

Several studies have demonstrated the stability of dried biofuid spots during short and long term sampling. Kong *et al.* showed that 10 metabolites randomly selected across the analysis appeared to be stable in dried blood spots (DBS) for up to 48 h at both -20 and 25 °C (Kong, Lin *et al.* 2011). Brauer *et al.* compared DBS with serum and

plasma and found increased concentrations of long-chain acylcarnitines and a decrease of arginine concentrations in DBS at room temperature or 5 °C, however, storage at -80°C for 3 months revealed no significant change (Brauer, Leichtle *et al.* 2011). Mitropoulos *et.al* suggested that long-term DBS sample stability is limited unless the cards are stored at, at least, -20 °C and preferably at -80 °C (Michopoulos, Theodoridis *et al.* 2011). Regarding IVE paper-spot method reproducibility, whilst the intra-batch reproducibly of IVE paper-spot was assessed in Chapter 2, the inter-batch storage stability during one week will be evaluated in this study.

In the present chapter, high-resolution UHPLC-Q-Exactive™ Orbitrap-MS analysis was performed to investigate 61 urine samples from sports which were prepared by a conventional method and IVE dried paper-spot method. Salbutamol, a prohibited substance in sports (when in excess of 1000 ng/mL in urine) was used as a model drug for the evaluation of dried urine spots. Then the paper spots stability after a one-week freeze-thaw cycle was assessed. Univariate and multivariate statistics were implemented to interpret the data output obtained from the UHPLC-MS. The workflow of this study is shown in **Figure 4-1**.

The aims of this chapter are: 1) To compare of paper-spot method with conventional liquid urine sampling method; 2) To appraise the paper-spot method for metabolomics discrimination of salbutamol “users” and “non-users”; 3) To evaluate the short-term stability of the paper-spot method.

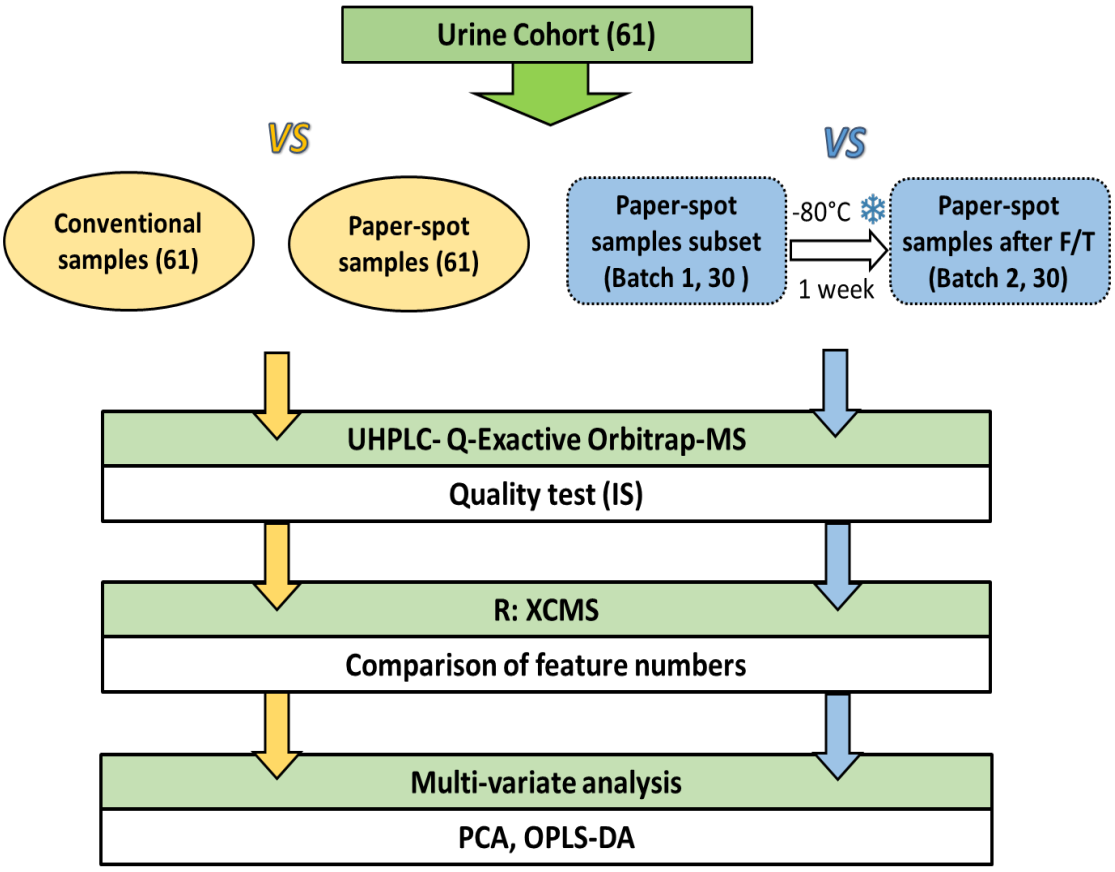

Figure 4-1 Metabolomics strategy workflow of this chapter. Firstly the comparison was conducted between conventional and paper-spot methods, the data analysis focused on three areas of interest: 1) Data quality test using internal standard (IS); 2) Feature number comparison after peak picking and alignment; 3) multivariate analysis after normalization. Secondly, 30 samples from the paper-spot method were randomly chosen and stored in -80°C for one week and were defrosted. Data acquired before and after the freeze-thaw cycle (F/T) (Batch 1 and Batch 2 respectively) were compared following the aforementioned procedures.

4.2 Methods

4.2.1 Chemicals and materials

All solvents used were HPLC grade. Acetonitrile and methanol were obtained from Fisher Scientific (Loughborough, UK). Formic acid (99–100 %) was purchased from VWR (Leicestershire, UK). Ultrapure water (18 MΩ · cm) was obtained from an ultra-pure water system (Elga, UK).

4.2.2 Urine samples

The samples from antidoping tests were acquired after professional sport competition and came from diverse demographics at random times of the day. A total of 61 urine samples were obtained from 61 different athletes, who had indicated their consent for research on their samples, with 30 salbutamol detectable samples (DET, 10 - 1000 ng/mL, 19 males and 11 females) and 31 salbutamol non-detectable samples (NON-DET, less than 10 ng/mL, 20 males and 11 females). The numbers of samples for endurance sports were matched in both DET and NON-DET groups. The specific gravity was measured by a calibrated DM40 densitometer (Mettler-Toledo International, Switzerland). All urine samples were aliquoted and stored in polypropylene tubes at -20 °C for at least three months and anonymised prior to analysis to comply with the ethical requirements of the World Anti-Doping Agency (WADA).

4.2.3 Sample preparation

A pooled sample from 20 urine samples with an equal number of DET and NON-DET was used as a quality control (QC).

In the conventional method, all the urine samples were centrifuged at 6,000 r.c.f for 15 minutes to remove any particles. An aliquot of 50 µL of supernatant was diluted with 200 µL ultrapure water previously spiked with internal standard (IS) containing D3-salbutamol at 500 ng/mL. The solution obtained was pipetted into amber HPLC vials with inserts.

In the paper-spot method, an aliquot of 2µL urine sample was spotted on each sample paper card (Whatman™ protein saver 903) resulting in the diameter of the urine spot less than 6 mm, and dried in ambient temperature for half an hour. These procedures were then repeated 4 times. A total volume of 10 µL urine was spotted on the same position of each paper. Spots with a diameter of 6 mm were punched out of the paper cards and introduced into amber HPLC vials. A volume of 50 µL 50 % methanol in ultrapure water containing 500 ng/mL IS was added to each vial containing the urine spots. All the vials were vortexed for 10 minutes before being centrifuged at 6,000 r.c.f. for 15 minutes. Then a volume of 10 µL sample in each vial was injected into the LC-MS system. All the samples were randomized and QCs were inserted after every 9 samples throughout the whole run.

4.2.4 Inter-batch stability

To make a Batch 2, 30 paper-spot samples (15 DET and 15 NON-DET) and 7 QCs were randomly chosen after LC-MS run and stored in -80 °C for one week. These

sample vials were then defrosted and analysed using the same conditions stated in section **4.2.3**.

4.2.5 UHPLC–HRMS analysis

For a detailed description of chromatographic separation, refer to section **3.2.4**.

Detection was performed by means of a Q-Exactive™ Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with a heated electrospray (HESI-II) ion source, operated in polarity switching mode. The instrument was calibrated before each batch of samples. Source conditions were as follows: sheath gas flow rate, auxiliary gas flow rate and sweep gas flow rate: 70, 10 and 0 arbitrary units respectively; capillary temperature: 320 °C; heater temperature: 350 °C; spray voltage: +3.75 kV (positive ionisation mode) and −3.00 kV (negative ionisation mode); lock mass: off. Nitrogen was used as both the source and the collision (HCD) gas. Data acquisition was performed at 70,000 resolution (FWHM). Each acquisition cycle included a full-scan acquisition in positive ionisation mode and a full-scan acquisition in negative ionisation mode. The AGC target was set to $1e^6$ and the scan range was *m/z* 100–1000 for all acquisition events. The resulting scan rate was 1.2 s.

4.2.6 Data processing and analysis

All raw data were collected using Thermo Xcalibur software. Internal standard (IS) peak area of each sample was calculated to process data quality test. Then relative standard deviation (RSD) of IS peak area was calculated.

All data were processed within XCMS in R and exported into SIMCA version 14 (MKS Umetrics AB, Sweden) for multivariate analysis. Data files (.RAW) collected in Xcalibur were converted by Proteomic Wizard to .MZXML format, before importing into freely available XCMS software. For a detailed description of XCMS settings, refer to section **3.2.5**. The resulting tables were readily exported to Microsoft Excel and feature numbers of both methods were obtained. Then the data collected from both positive and negative ionisation modes were normalized by the specific gravity (SG) of each sample prior to multivariate analysis. For a detailed description of calculation of normalization, refer to section **3.2.5**.

The data from both positive and negative ionisation mode were then normalized by the specific gravity of each sample, before importing into SIMCA 14, where it was subjected to multivariate data modelling, including pareto-scaled principal component analysis (PCA) and log-transformed orthogonal partial least-squares discriminant analysis (OPLS-DA) with corresponding S-plot analysis. Graphical outputs were prepared in SIMCA.

4.3 Results and discussion

4.3.1 Conventional method vs paper spots method

4.3.1.1 Overview of the chromatograms

As can be seen from the positive Base Peak Intensity (BPI) chromatogram of urine shown in **Figure 4-2**, the largest base peak intensity was reduced approximately by 30 % from conventional method to paper-spot method, and more significant reductions are observed at other time points such as 2.28, 3.27, 4.50-4.80 and 6.45-6.84 min.

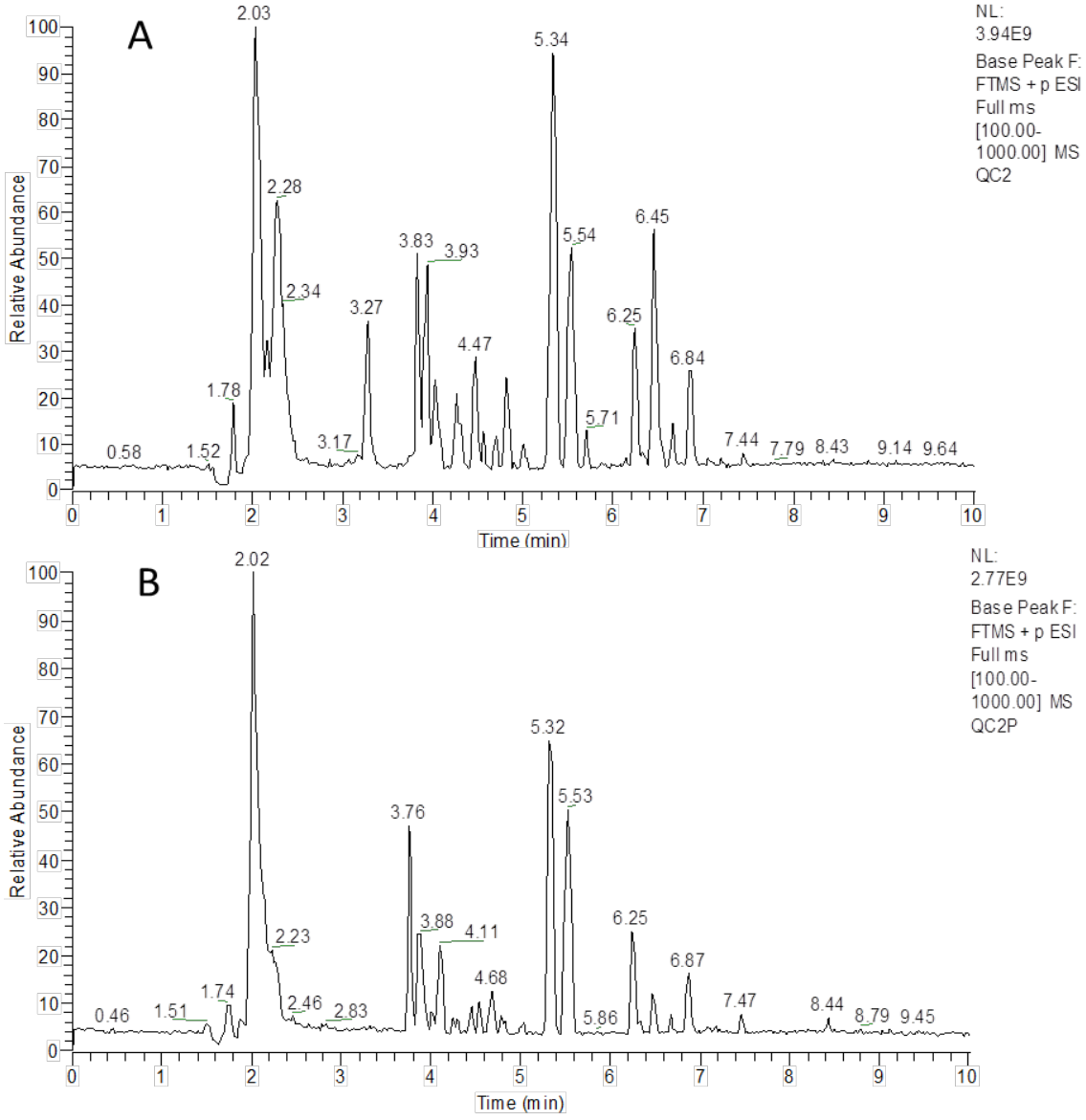

Figure 4-2 Representative BPI chromatograms in positive ionisation mode. The chromatograms were normalized to the largest peaks and y-axis is relative abundance level. A, conventional sample; B, dried urine spots. NL, normalized level, represents the largest peak ion count.

For negative mode, around 20 % reduction of the intensity of the largest peak is observed after spotting, and more noticeable decreases are observed at time points such as 1.96, 2.91, and 6.14 min, etc. (**Figure 4-3**).

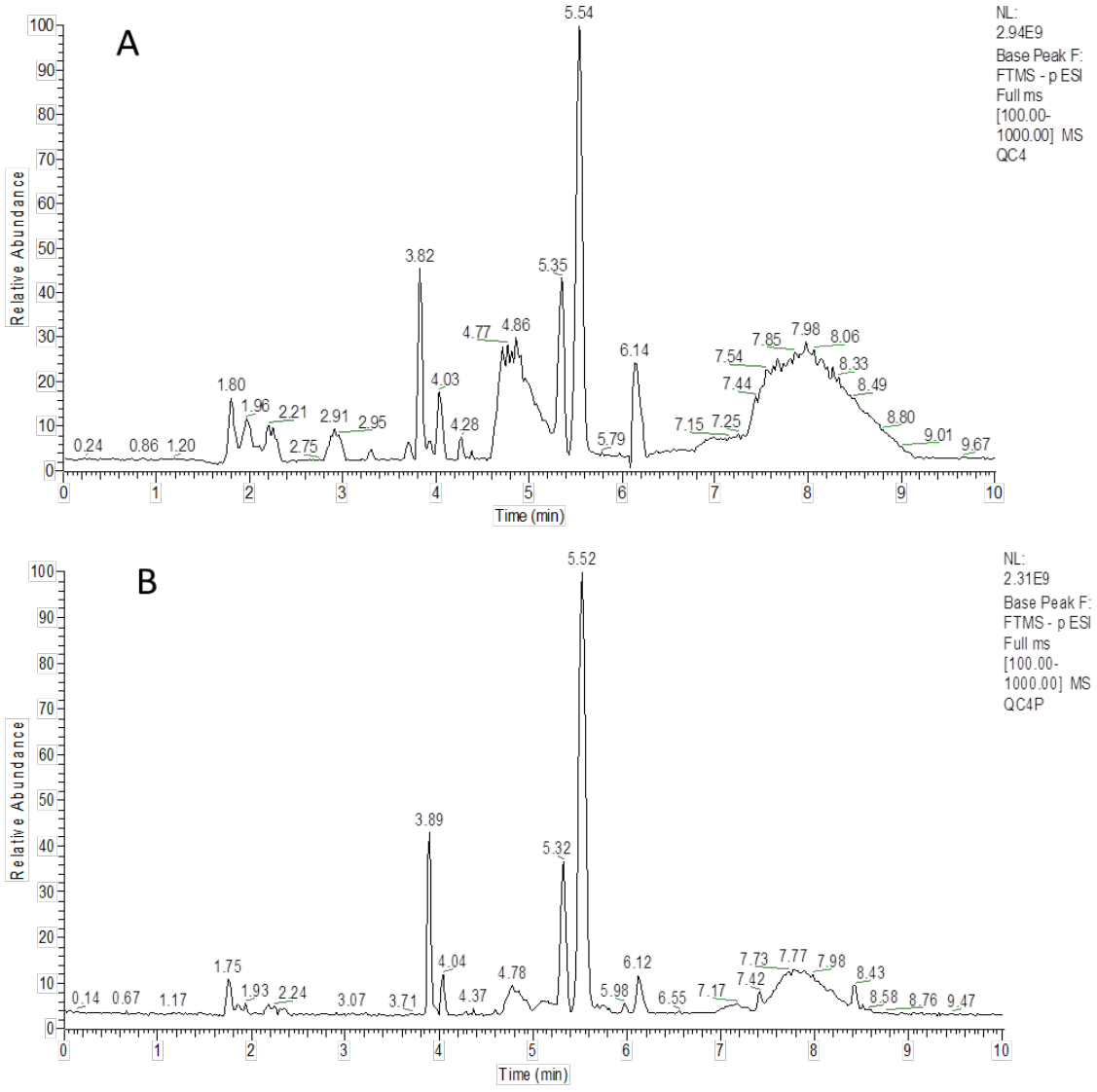

Figure 4-3 Representative BPI chromatograms in negative ionisation mode. The chromatograms were normalized to the largest peaks and y-axis is relative abundance level. A, conventional sample; B, dried urine spot. NL, normalized level, represents the largest peak ion count.

4.3.1.2 Comparison of feature numbers

Pre-processing steps were applied in parallel to both sampling methods (N=61 in each method) and the features pertaining to each were extracted. The following table shows the total numbers for comparison (**Table 4-1**):

Table 4-1 Number of features generated in R: XCMS of two methods in two ionisation modes (N = 61 in each method)

	Conventional method	Paper-spot method
Negative mode	2,205	2,054
Positive mode	2,758	2,262

The data were processed in R: XCMS and a total of 4,963 and 4,316 features acquired in the conventional and paper-spot methods were extracted, respectively. It can be observed in **Table 4-1** that the positive ionisation mode yielded more features compared to the negative ionisation mode, and the conventional method generated more features than the paper-spot method. The procedure of spotting urine samples on a paper and extraction before injecting into MS could have led to feature intensity reduction and even loss. In the sample-spotting step, some metabolites with lower boiling points could evaporate and, in the extraction step, some metabolites might stick on the sampling paper.

After spotting, there was a 7 % feature number reduction for the negative ionisation mode, compared to the 18 % reduction in positive mode, and this is consistent with the base peak chromatograms (**Figure 4-2 and 4-3**). However, a paper published by

Michopoulos *et al.* showed a significant reduction in feature number for spotted samples in negative ionisation mode, with only 30 % of the features from liquid samples were detected in the spotted samples (Michopoulos, Theodoridis *et al.* 2010). These contradict results were not surprising, probably being caused by: 1) The different sample preparation and extraction methods: in this study IVE method was used on the entire sample spot to preserve the metabolites; while in their study, multiple sample cores were extracted using 20 % aqueous methanol, and the extraction supernatants were evaporated in 40 °C and resuspended, which could probably cause loss of volatile metabolites; 2) Different detector: in our method, a Q-Exactive™ Quadrupole-Orbitrap mass spectrometer was used, while their experiments were conducted using Q-TOF Micro mass spectrometer. The different resolution and sensitivity of the instrument could possibly lead to different feature numbers as in paper-spot method some ion intensity could dramatically be reduced. 3) Other experimental differences may also account for the different outcomes, such as column, UHPLC instrument and method, data pre-processing method, etc.

4.3.1.3 Data quality test

Data quality test was carried out to assess the variance caused by sample handling and analytical instruments, via the descriptive statistics of the IS peak areas in the raw data from both the conventional and paper-spot methods (**Table 4-2**):

Table 4-2 Descriptive parameters calculated from the peak areas of IS in the two methods (N = 61 in each method)

	Conventional method peak area	Paper-spot method peak area
MEAN	$2.23e^{9}$	$2.71\ e^{9}$
SD	$5.14\ e^{8}$	$4.79\ e^{8}$
% RSD	23.0	17.7

It can be observed from **Table 4-2** that the paper-spot method showed higher precision in the quality test as the RSD of the IS in paper-spot method was lower than that in the conventional method. Peak signal stability is influenced by factors such as the type of molecule being detected, absolute concentrations, matrix effects (Ellison and Williams 2012) and systematic errors introduced during the analysis, and the lower RSD in the paper spots might have resulted from the paper-spot samples being cleaner, since some molecules that affect analysis, such as remaining proteins might have stayed on the paper. The features that could cause the higher bias (e.g. volatile, unstable analytes) were not studied in this analysis, salbutamol is a relatively easy molecule to analyse, and its behavior cannot be extrapolated to other metabolites.

If we compare these data with FDA quantitation standards (FDA 2013) the RSD should be lower than 15 % so at the moment neither sampling method would pass the criteria for quantification. When sample volume is limited or near LOD (limit of detection) the criteria can be extended to 20 % variation, considering that our samples were only 10 μL with no pre-concentration and clean-up step. In a metabolomic study, thousands of metabolites are screened in one-go, thus quantification rules are not yet

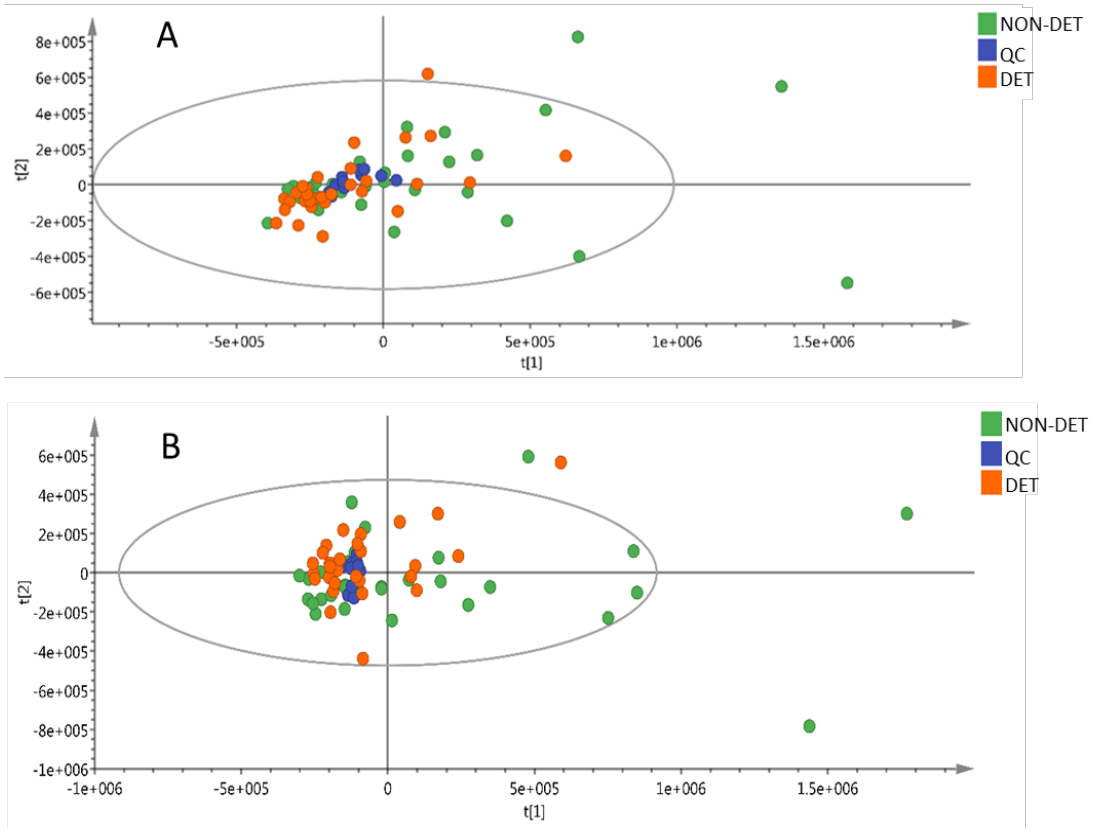

Figure 4-4 PCA score scatter plots of conventional method (A) and paper-spot method (B). Data were from positive ionisation mode.

Multivariate analysis results are shown in **Figure 4-4, 4-5** and **4-6**. PCA score plot is an overview of the entire multivariate data showing observations and their relationships. The "pooled" quality control (QC) urine samples were used to provide a representative mean sample. By detecting the distribution of QC samples in PCA plots, the quality of the data corresponding to the metabolomics method applied can be assessed. PCA plots of both conventional and paper-spot methods (**Figure 4-4**) show that the QC groups cluster together, which indicate good overall data reproducibilities in both methods.

OPLS-DA was performed to distinguish two classes of interest among 30 DET and 31 NON-DET samples. After log-transformation, removal of outliers and excluding variables with VIP (variable importance for the projection) value <1, final OPLS-DA models were rebuilt (**Figure 4-5**). Both OPLS-DA modelling can demonstrate DET isolation from NON-DET.

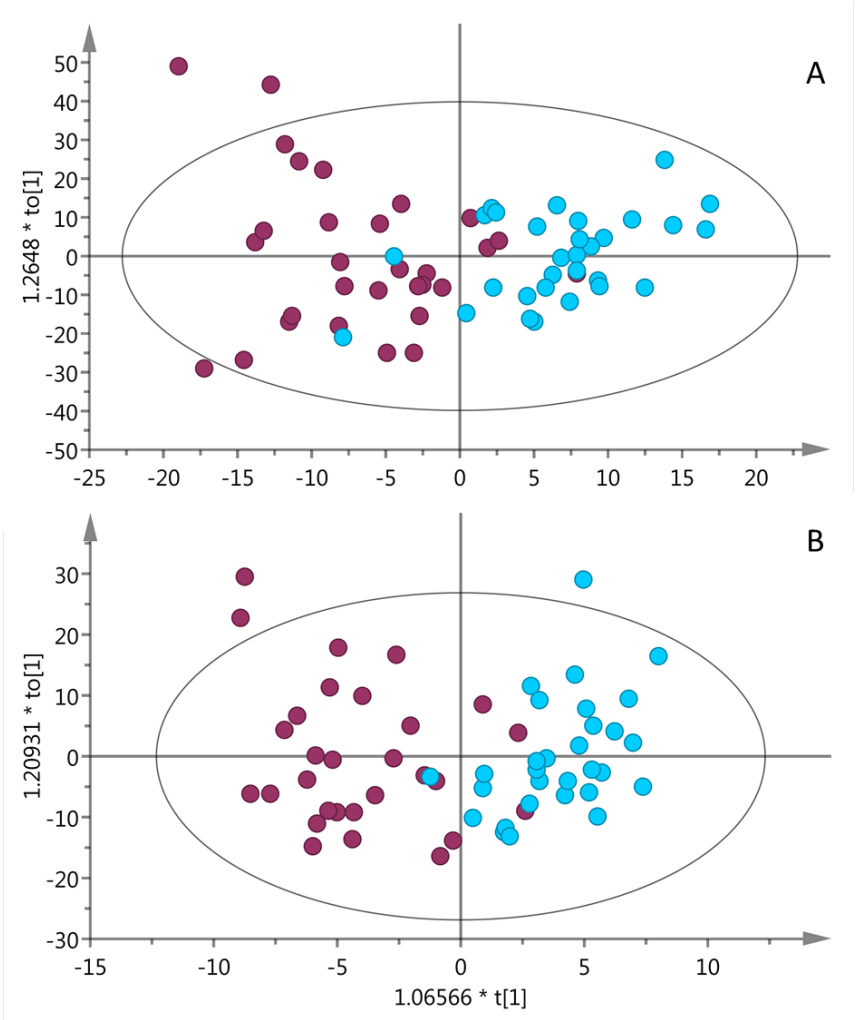

Figure 4-5 OPLS-DA score plots depicting the discrimination of DET and NON-DET samples. A, conventional method; B, paper-spot method. Blue, DET; Purple, NON-DET. Data were from positive ionisation mode.

Figure 4-5 B is a plot of OPLS-DA modelling of all urine samples in the paper-spot method, as analysed by UHPLC-Q-Exactive MS in the positive ionisation mode. The R^2X, R^2Y, Q^2 and CV-ANOVA values of each model are shown in **Table 4-3**. The algorithm was able to clearly discriminate the urine samples into 2 groups corresponding to DET and non-DET samples in the paper-spot method showing a CV-ANOVA p-value = 2E-4. It also shows a clear separation and a significant model when such analysis was performed in the conventional method (**Figure 4-5 A, Table 4-3**), although not as discriminating as in paper-spot method. The paper-spot method model explains more of x-variables (R^2X = 0.483) and have a better model fit (R^2Y = 0.697) and prediction (Q2 = 0.385) than the conventional method, which might have resulted from the paper-spot samples being cleaner as mentioned in section **4.3.1.3**. These indicate that the paper-spot method could possibly work better in metabolomics for the prediction of some doping substances such as salbutamol.

Table 4-3 Some parameters for the OPLS-DA models of the two methods

	Conventional method	Paper-spot method
R^2X	0.314	0.483
R^2Y	0.582	0.697
Q^2	0.266	0.385
CV-ANOVA (p-value)	1.7E -3	2E -4

Metabolic correlations between all the discriminating metabolites were constructed in the corresponding correlation S-plots (**Figure 4-6**). S-plots of both conventional and

paper-spot methods show that the most significant feature with the largest correlation value to DET group is salbutamol. This is an expected result provided that the LC-MS analysis was appropriate to detect salbutamol and that the data pre-processing and modelling steps were built correctly. The P correlation value (p (corr)) of the conventional method (p (corr) = 0.75) is in the same range as that in the paper-spot method (p (corr) = 0.73). These indicate that both methods were adequate to discriminate our model drug or a potential biomarker of similar characteristics using urine metabolomics.

The sensitivity of paper spots can decrease according to **Figure 4-2**, yet it is sufficient to detect salbutamol with a wide concentration range from 10 – 1000 ng/mL. However, we also concern about the sensitivity of the endogenous compounds altered upon salbutamol administration as well as other prohibited drug with lower decision limits, and whether or not they can be detected using paper spots were not investigated in this chapter.

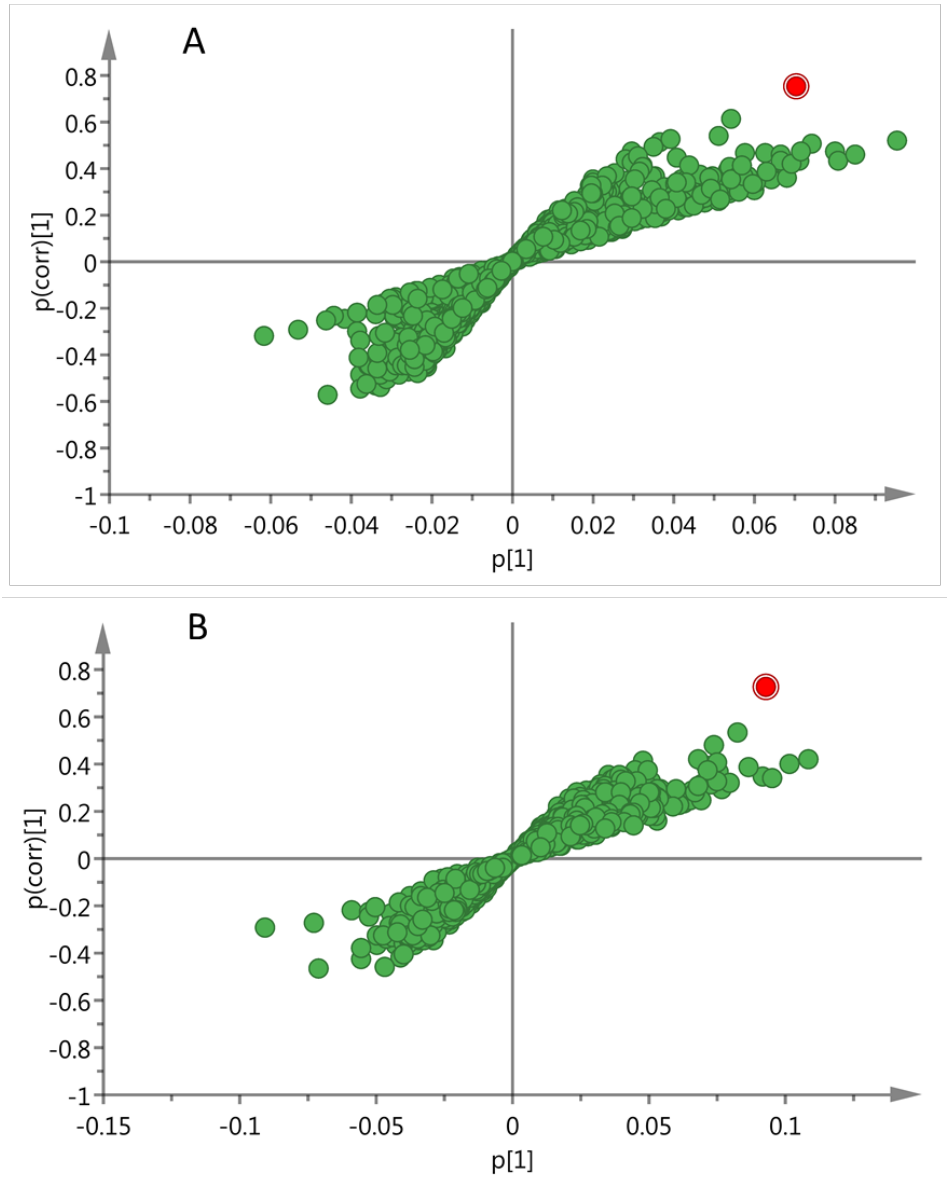

Figure 4-6 S-plot depicting salbutamol (marked in red) as the most significant feature of in conventional method. A, salbutamol p(corr) = 0.75 in conventional method; B, salbutamol p (corr) = 0.73 in paper-spot method.

4.3.2 Paper-spot inter-batch reproducibility after a F/T cycle

4.3.2.1 Comparison of feature numbers

Thirty samples were randomly selected and subjected to UHPLC-MS analysis for a second time after a single one-week -80 °C freeze-thaw cycle. The two raw data batches were processed separately in XCMS using the parameters aforementioned in the method section **4.2.6**. The results showed high similarity in feature numbers between the two batches, with slightly less features in positive ionisation mode for Batch 2 (**Table 4-4**). This indicated that there was good stability in metabolite numbers between batches. The non-significant reduction of the feature number could be due to the freezing, storage and defrosting procedures, or merely system variability.

Table 4-4 Number of features generated in R: XCMS of two batches in two ionisation modes (N = 30 in each batch)

	Batch 1	Batch 2 (after F/T)
Negative mode	2,081	2,070
Positive mode	2,132	2,053

4.3.2.2 Data quality test

The peak area of internal standard was quantified in the raw data in both paper-spot batches, and **Table 4-5** shows the descriptive statistics:

Table 4-5 Descriptive parameters calculated from the peak area of IS in the two methods (N = 30 in each batch)

	Batch 1	Batch 2 (after F/T)
MEAN	2.99 e^9	2.85 e^9
SD	5.10e^8	3.00 e^8
% RSD	17.0	10.5

The IS of the two batches showed similar intensity as we can expected. T-test showed there is no significant difference between Batch 1 and Batch 2 (p >0.05). D3-salbutamol is a relatively stable compound, and its behaviour cannot be extrapolated to other metabolites. However the RSD of 10.5 % shows good overall system stability for Batch 2.

4.3.2.3 Multivariate analysis

The two batches of raw data were processed together in XCMS, before exporting to SIMCA to perform PCA analysis. The PCA score plot in **Figure 4-7** shows two batches of samples evenly spread in the plot, which indicates that there was no significant difference caused by a one-week freeze-thaw cycle. The QCs of two batches cluster and mix together in the centre, showing an acceptable intra-batch and inter-batch data stability. Moreover, the two batches are closely paired together, and noticeably two batches of the outliers, S11 (from DET group) and N22 (from NON-DET group), cluster too, which revealed the high similarity between the two batches.

Here we studied the stability during a one-week freeze-thaw cycle but longer term stability of IVE paper-spot should be thoroughly investigated, and the metabolomics strategy proposed here is also applicable. In addition, the stability of different types of substances including various categories of drugs and endogenous compounds should be assessed when using the IVE paper-spot sampling method. More and more metabolomics projects use sample numbers in the 1000s, and that would translate to months of storage which in turn could bring some unexpected stability effects.

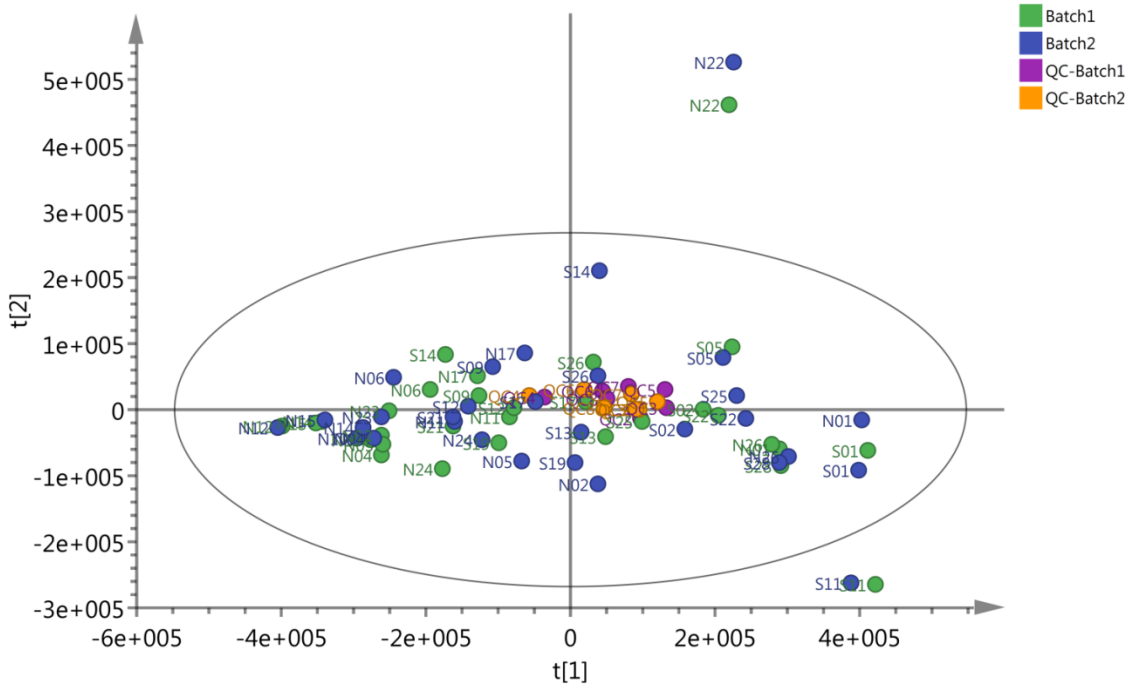

Figure 4-7 PCA score scatter plots of 2 batches of 30 samples and 7 QCs. Samples with names started with "S" were from DET group, while samples with names started with "N" were from NON-DET group. The QCs of two batches cluster and mix together in the centre, indicating an acceptable intra-batch and inter-batch data stability.

4.4 Conclusions

Urine metabolomics analysis of 61 urine samples was performed to evaluate conventional urine liquid sampling and IVE of dried paper spots using UHPLC-Q-Exactive™ Orbitrap-MS. Two groups (DET and NON-DET) were created in each sampling procedure according to whether they contained salbutamol, which was used as a model non-endogenous drug metabolite.

For the comparison between the two sampling methods, there were slightly less features in paper spots than that with conventional urine samples, despite the base peak intensity dropped by more than 30 % in positive ionisation mode and 20 % in negative ionisation mode consequentially after spotting and extraction. However, lower variation (17.7 % versus 23.0 %) was found in the paper-spot method showing higher precision. Furthermore, OPLS-DA models showed better discrimination capacity of variables for DET and NON-DET groups in the paper spots than using the conventional method (CV-ANOVA p-value = 2E -4 versus 1.7E -3). The S-plot of both methods indicated both methods were adequate to discover the main discriminating feature salbutamol (with correlation values of 0.73 and 0.75 for the positive group) in urine using metabolomic analysis.

For the evaluation of IVE paper-spot inter-batch reproducibility, two batches of LC-MS data were accessed and compared. The results showed good inter-batch stability and little information loss after a one-week -80 °C freeze-thaw cycle, and multivariate analysis indicated a close relationship between the two batches.

To summarize, IVE paper-spot sampling can cause some information loss but it proved to be robust and very similar to conventional sampling when used for urine metabolomics, and it could be a good alternative when sample volumes are limited, such as in studies when many "omics" analyses are required from one sample. However the long-term reproducibility of spots still need to be evaluated, especially when bearing with other doping agents or endogenous metabolites that are not as stable as salbutamol.

In the next chapter, we will apply the metabolomics strategy to anti-doping application. As the paper-spot method risks a loss of sensitivity and there are sufficient sample volumes at this time, conventional liquid samples will still be used.

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Chapter 5 Metabolic phenotyping of urine samples to discover surrogate markers for GHB administration

5.1 Introduction

A small molecule phenotyping strategy for HRMS-based metabolomics was developed in Chapters 2-4, using both conventional and paper-spot sampling methods. In this and the next chapters, the strategy will be applied to the anti-doping field using endogenous and exogenous model compounds, respectively, to finally test the general hypothesis. Since the paper-spot method risks a loss of sensitivity and there are sufficient sample volumes at this time, conventional liquid samples will still be used.

One of the main challenges in anti-doping control is the ever increasing number of endogenous and exogenous compounds to be screened routinely. New drugs appear on the market every day and athletes quickly switch to new substances to avoid being caught. As stated in Chapter 1, anti-doping laboratories in recent years have started modifying their screening approach from targeted data acquisition (e.g. SRM) to total data capture using high resolution mass spectrometry instruments(Virus, Sobolevsky *et al.* 2008, Peters, Oosterink *et al.* 2010, Vonaparti, Lyris *et al.* 2010, Musenga and Cowan 2013, Thevis, Kuuranne *et al.* 2014). This permits total data capture and eliminates the requirement for *a priori* selection of the compounds to be targeted. Though this adds tremendous flexibility to the methods and enables retrospective analysis of samples, the data processing step still depends on a targeted approach, where “targeted” extracted ion chromatograms are generated from the “untargeted” acquired data.

Biomarker screening from metabolomic profiling is an alternative that has the potential of spotting both up- and down-regulation of metabolites and creating predictive models for individual or group case studies. Approaches that do not target the doping compound would be very valuable; surrogate markers could be identified and quantified where necessary. Depending on the surrogate's specificity this could lead to, for example, a prolonged detection time, or improved detectability for substances at very low concentrations and/or which present challenges being detected. At present, the metabolomic biomarker strategy has been applied in a variety of fields, including diagnostics, patient stratification, drug development and clinical chemistry (Lindon, Holmes *et al.* 2007, Nicholson and Lindon 2008, Patti, Yanes *et al.* 2012, Sen, Wang *et al.* 2013, Jung, Jung *et al.* 2014, Lindon and Nicholson 2014, Mathe, Patterson *et al.* 2014). It is of particular interest in areas like doping control, as new approaches are needed to fight the ongoing development of performance-enhancing methods (Riedmaier, Becker *et al.* 2009, Teale, Barton *et al.* 2009, Guha, Erotokritou-Mulligan *et al.* 2014). So far, the biomarker approach of the combined detection of insulin-like growth factor-I (IGF-I) and procollagen type III amino-terminal propeptide (P-III-NP) for testing growth hormone (GH) misuse has been approved by the WADA and has led to the detection of a number of athletes misusing GH (Powrie, Bassett *et al.* 2007, Holt 2013).

In this chapter, the previously-developed metabolomic strategy is applied to samples from a clinical trial for GHB administration, with the aim of finding the biomarker of administrated gamma-hydroxybutyrate (GHB), which is used as an endogenous model compound.

Gamma-hydroxybutyrate (gamma-hydroxybutyric acid, GHB, molecular structure showed in **Figure 5-1**), an endogenous, naturally occurring compound in mammals, has gained increased public attention for its illicit use, including recreational use and drug-facilitated sexual assaults (DFSA). It can be used alone or mixed with other substances in beverages due to its colourless and odourless liquid state (Freese, Miotto *et al.* 2002, Kintz, Cirimele *et al.* 2003, Castro, Tarelho *et al.* 2016).

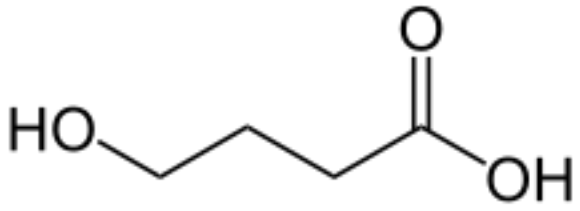

Figure 5-1 The molecular structure of GHB.

GHB was initially used in Europe as a general anesthetic and sleep aid, but usage was ceased because of its insufficient anesthetic potency and unpredictable duration of action (Tunstall 1968, Margulis, Andreyev *et al.* 1975). It was also once marketed in health food stores in the U.S., as a growth hormone releasing agent to stimulate muscle growth. Again, it was taken off the shelves and has been banned since the late 1990s in most countries. Today it is only allowed for the treatment of narcolepsy and rare alcoholism (Mayer 2012, Keating 2014, Caputo, Mirijello *et al.* 2015). However, GHB as well as Gamma-butyrolactone (GBL) became popular substances of abuse because of the euphoria and hallucinogenic effects, branding these molecules with the name "liquid ecstasy". Moreover, it is also used as a doping agent by sports athletes due to a growth hormone secretion elevating effect (Van Cauter, Plat *et al.* 1997, Volpi,

Chiodera *et al.* 1997). In addition to that, strong sedative and amnesic properties of the drug further propel the abuse of GHB by criminals in DFSA: it can be spiked in drinks of unsuspecting victims and its sedative effect is exploited for "date-rape" criminal offenses.

From the forensic perspective, however, to prove the ingestion of GHB, surreptitious or deliberate, is rather difficult due to its endogenous nature and rapid metabolic elimination rate (plasma $t_{1/2}$ = 20 - 40 minutes), which leads to levels of GHB in urine back to basal concentration during 4-12 h after ingestion and even shorter time in blood (Brenneisen, Elsohly *et al.* 2004, Abanades, Farre *et al.* 2006, Brailsford, Cowan *et al.* 2012, Liechti, Quednow *et al.* 2016). This brief window makes proof of administration problematic in most rape cases.

Urine is the most used specimen matrix currently for GHB testing, the cut-off value of 10 mg/L in human urine is typically considered to distinguish between endogenous concentration and any concentration consistent with administration (Shima, Miki *et al.* 2005, LeBeau, Montgomery *et al.* 2006, Brailsford, Cowan *et al.* 2010, De Paoli, Walker *et al.* 2011). Hair (Jagerdeo, Montgomery *et al.* 2015, Vaiano, Serpelloni *et al.* 2016) and saliva (De Paoli, Walker *et al.* 2011, Mazina, Saar-Reismaa *et al.* 2015) specimen analysis using GC-MS, LC-MS/MS and CE have been reported by research groups.

The metabolism of GHB occurs via GHB dehydrogenase to succinic semialdehyde (SSA) and then subsequently to succinic acid and gamma-Aminobutyric acid (GABA) (Castro, Dias *et al.* 2014). Recently, glucuronide (Petersen, Tortzen *et al.* 2013) and

sulfonated (Hanisch, Stachel *et al.* 2016) metabolites of GHB were reported. Although the mechanism of action of GHB is still not fully elucidated, it is generally accepted that GHB affects the balance of neurotransmitters (Drasbek, Christensen *et al.* 2006).

To extend the detection window, some studies focused on the discovery of surrogate markers of GHB. Larson *et al.* found that epiregulin and Pea-15 protein levels increased in a GHB dosed mouse model by means of transcriptomic and proteomic approaches, and suggested that these two proteins could be used as surrogate markers in the indirect detection of GHB that may extent the detection window from 12 to 48 hours (Larson, Putnam *et al.* 2007). Using metabolomics, Nasrallah *et al.* incubated guinea pig cortical tissue slices with GHB and observed various metabolic responses on different concentrations of GHB, and suggested potential target receptors for further investigation (Nasrallah, Maher *et al.* 2010). However, no study has been reported on untargeted metabolomic approach to find biomarkers of GHB using human samples so far.

The objective of this chapter is to find a biomarker of GHB administration in human urine samples using an untargeted metabolomic strategy in order to aid the short detection window of GHB. The samples from volunteers were obtained from a small clinical trial that looked into the pharmacokinetics of GHB (Brailsford, Cowan *et al.* 2012). Potential biomarkers of GHB were mined by questioning the data in different ways accompanied by a relevant statistical data processing tool.

The study design is depicted in **Figure 5-2**:

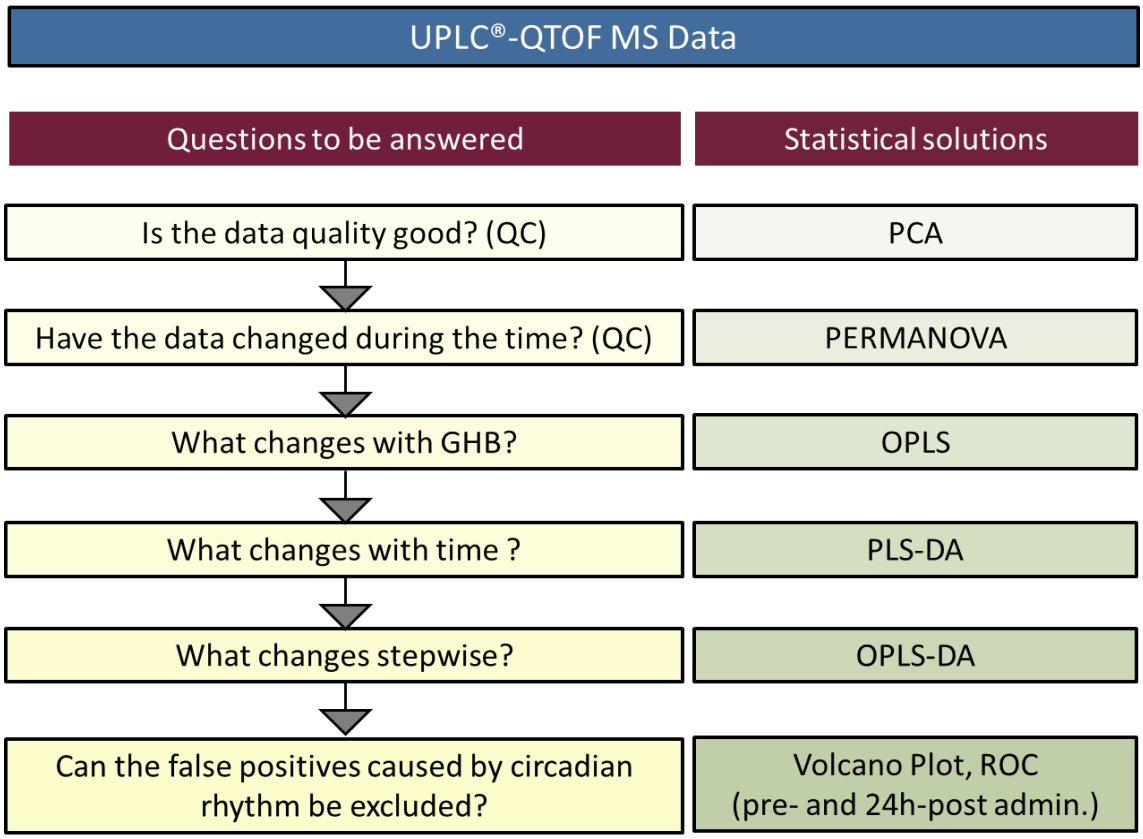

Figure 5-2 Data treatment workflow for longitudinal study of GHB administration. PCA: principal component analysis; PERMANOVA: permutational multivariate analysis of variance; OPLS (-DA): orthogonal partial least-squares (discriminant analysis); PLS-DA: partial least-squares discriminant analysis; ROC: Receiver Operating Characteristic; QC: quality control; admin.: administration.

5.2 Methods

5.2.1 Chemicals and materials

GHB (sodium salt, Lot analysis = 99.7% purity), deuterated GHB-d6 (4-hydroxy-2,2,3,3,4,4-hexadeuterobutyric acid sodium salt) supplied as 1 mg/mL stock solution in methanol, LC-MS grade formic acid were purchased from Sigma-Aldrich Company

(Poole, U.K.). LC−MS grade water and acetonitrile were purchased from VWR International (Fontenay-sous-Bois, France). All sample extractions were performed in 2mL microcentrifuge tubes (from Eppendorf, Hamburg, Germany). Amber glass HPLC vials with fixed 0.3 mL inserts were from Chromacol (Welwyn Garden City, U.K.).

5.2.2 Volunteers and urine samples

Samples were from a previous GHB pharmacokinetics study (Brailsford, Cowan *et al.* 2012) taken from 12 healthy volunteers (6 females and 6 males), see **Figure 5-3**.

Volunteer recruitment

Ethical approval for the GHB administration study was obtained from our institutional research ethics committee (approval number CREC/06/07-30). Written informed consent was obtained from the volunteers (six men and six women). Males had a mean age of 25 years (21–36 years) and a mean body mass index (BMI) of 23.7 kg/m^2 (21.7– 27.1 kg/m^2). Females had a mean age of 26 years (22– 32 years) and BMI of 23.0 kg/ m2 (19.5– 25.9 kg/m^2). Prior to the study, volunteers were assessed to be in good health. Exclusion criteria included a history of liver disease, succinic semi-aldehyde dehydrogenase deficiency, and currently breast feeding. All volunteers tested negative for the current use of sedatives, recreational drugs, and pregnancy (females only) by analysis of a urine sample collected one week before administration of GHB.

Study design

On the day of study, volunteers were asked to finish a light breakfast by 7:30 a.m. before arriving at the secure study suite. At 10:00 a.m., a single dose (25 mg/kg body weight) of GHB was administrated in the form of the pharmaceutical preparation Xyremw (sodium oxybate, 500 mg/L). The mean dose was 1.8 g (as the sodium salt) and ranged from 1.4 to 2.6 g (equivalent to 1.2 –2.1 g GHB). The preparation was diluted with 60 mL water prior to administration as directed by the manufacturer. Urine samples were taken for 13 time points at –10 min, 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, and 30 h. The time point numbers and their corresponding time are shown in **Table 5-1**.

Table 5-1 The time point numbers and their corresponding time of urine samples.

Time point No.	T1	T2	T3	T4	T5	T6	T7
Time	-1 week	-10 min (0 h)	1 h	2 h	4 h	6 h	8 h
Time point No.	T8	T9	T10	T11	T12	T13	T14
Time	10 h	12 h	14 h	16 h	20 h	24 h	30 h

Sample storage

The volunteers were asked not to void any urine at times other than those specified up to 30 h post-administration, during which time they were monitored. Samples were stored without preservative at -20 °C until analysis.

5.2.3 Sample preparation

A pooled sample was used as a quality control (QC). All the urine samples were centrifuged at 6,000 r.c.f for 10 minutes to remove any particles. An aliquot of 100 µL of supernatant pipetted into amber HPLC vials with inserts. GHB-d6 stock solution was diluted by LC-MS grade water to 20 µg/ mL to make an IS solution and 80 µL of the IS solution was added into each vial. The QCs were inserted after every 10 samples throughout the whole run.

5.2.4 Analytical Setup

For a detailed description of LC-MS instrument and column, refer to section **2.2.4**. Urine extract (5 µL) was injected into the LC-MS system. Separation was performed at 25 °C with a flow-rate of 0.2 mL/min, solvent A composed of 0.1% formic acid in water, and solvent B composed of 0.1% formic acid in acetonitrile. Metabolites were eluted using the following gradient elution method: from 0.5 % B at 0 min to 50 % B in 8 min, increasing to 100% B in 11 min, returning to initial conditions in 0.5 min, maintained at initial conditions for 2 min. MS analysis was completed in separate positive and negative mode experiments. In both modes, all analyses were acquired in LockSpray mode to ensure accuracy and reproducibility; leucine-enkephalin was used as a lock mass (*m/z* 556.2771) at a concentration of 500 ng/mL and a flow rate of 10 µL/min. Data were collected in the centroid mode over the *m/z* range of 80−1000 with an acquisition time of 0.1 s a scan. In positive ion mode, the XEVO® QTOF was operated with a capillary voltage of 3.2 kV and a cone voltage of 35 V. The extraction cone was set to 2.0 V. The desolvation gas flow was 500 L/h and maintained at 400

°C. The source temperature was 120 °C, and the cone gas was set to 50 L/h. In negative ionization mode, the XEVO® QTOF was operated with a capillary voltage of −2.6 kV and a cone voltage of 45 V. The extraction cone was set to 2.0 V. The desolvation gas flow was 800 L/h and maintained at 350 °C. The source temperature was 120 °C. All data were collected using Waters MS^e technology, at two constantly interchanging energies. For a detailed description of MS^e technology, refer to section **2.2.4**.

5.2.5 Data preprocessing

Data files (.RAW) collected in MassLynx were converted by Databridge to .CDF format before importing into freely available XCMS package in R. For peak picking, the following parameters were used: method = "matchedFilter", fwhm = 10, snthresh = 9, mzdiff = -0.001. For peak grouping, bw = 10, mzwid = 0.05 were used. After peak alignment to allow minor retention time variation and filling missing peaks, an output table of time-aligned detected features was exported with their retention time, mass to charge ratio (*m/z*), and intensity in each sample. A feature is any *m/z* signal maximizing at a specific retention time. The resulting tables were readily exported to Microsoft Excel and feature numbers were obtained in the Excel output tables.

5.2.5 Statistical analysis

The data from both positive and negative ionization mode were then normalized by the total void volume of each volunteer at each time point, before further analysis. The data from positive and negative ionization modes were combined, univariate-scaled

and log-transformed, before being subjected to orthogonal partial least-squares analysis (OPLS) with corresponding S-plot analysis in SIMCA 14 (UMetrics, Umeå, Sweden). PLS-DA and PERMANOVA were performed in mixOmics package in R. In stepwise analysis, orthogonal partial least-squares discriminant analyses (OPLS-DA) were used on grouped datasets in SIMCA 14. Volcano plot, ROC curve and boxplots were prepared in MetaboAnalyst (www.metaboanalyst.ca/) (Xia, Sinelnikov *et al.* 2015). Other graphical outputs were prepared in SPSS (IBM SPSS statistics, version 22).

5.2.6 Putative metabolite identification

The features that were observed to influence models the most were preliminarily identified using the human metabolome database (HMDB) (www.hmdb.ca/) (Wishart, Tzur *et al.* 2007, Wishart, Knox *et al.* 2009, Wishart, Jewison *et al.* 2013) and the equivalent METLIN (METaboliteLINk) database (https://metlin.scripps.edu/) (Smith, O'Maille *et al.* 2005).

Due to the high number of database hits generally returned by a database search, a number of rules were enforced in the identification database search in order to be as consistent as possible. These rules are listed and explained below:

- 30 ppm error

  The mass error of the search on the database searches was set to 30 ppm, as this was well within the accuracy of the Waters Xevo® MS and confirmed by our external calibration.

- Polarity

  The feature in each instance must realistically ionize in the polarity being observed. For example, GHB is generally only observed in positive ionization mass spectrometry.

- Human biology

  Features in the search must be possible metabolites in human urine. HMDB lists instances where the molecules have been extracted from human tissue or biofluids and previously reported in the literature.

- Retention Time

  Retention time and the matching chemical properties such as partition-coefficient (P) of each feature are considered, for example molecules with a similar polarity often elute in the same region of the chromatogram and metabolites with small or even negative log (P) elute early in the reverse phase chromatogram.

- MS^2 fragmentation

  As mentioned in section 5.2.4, accurate mass fragmentation data (MS^2) can be collected simultaneously with accurate mass precursor ion data. The identification will be more accurate if the fragment spectrum of the candidate can be confirmed in MS^2.

All of these rules were taken into consideration step-wise when completing the initial database search.

5.3 Results and discussion

A total of 5135 features were extracted after data pre-processing in XCMS, including 2399 features from the positive ionization mode and 2736 features from the negative mode.

5.3.1 Possible metabolites of GHB

It is overserved that urinary GHB reached highest concentration (C_{max}) at 1h post-administration and back to basal level in 4 h from the previous pharmacokinetic study (**Figure 5-3**, data not shown after 10 h post-administration).

All known metabolites of GHB were first checked in the raw data, including succinic semialdehyde (SSA), succinic acid, GABA, glucuronide and sulfonated GHB; however, none were detected in the chromatogram using fingerprinting. Therefore, the whole dataset after pre-processing were subjected to a battery of untargeted statistical analysis (**Figure 5-2**).

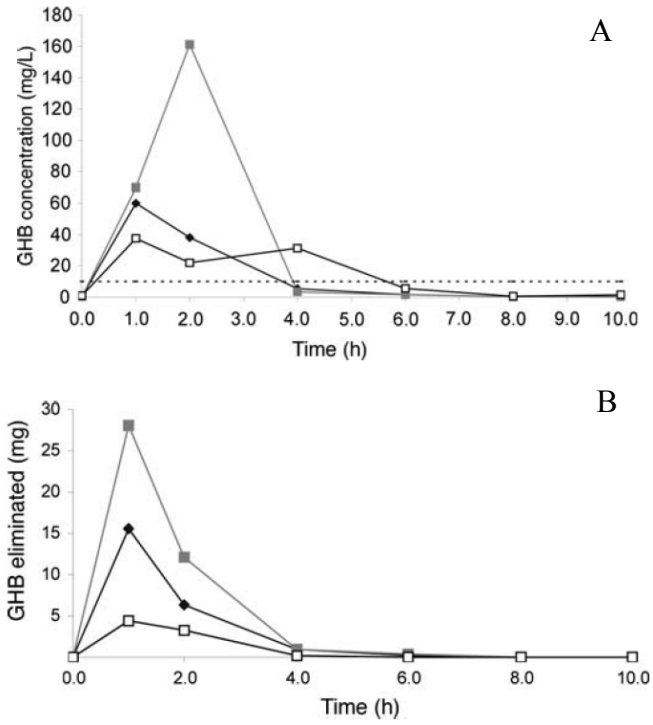

Figure 5-3 Urinary GHB behaviour in 12 volunteers following a 25 mg/kg oral dose for 10 h post-administration. A, Urinary GHB concentrations for 10 h post-administration. The dashed line represents 10 mg/L (discriminant limit). B, Total urinary GHB elimination for 10 h post-administration. Mean for 12 volunteers = ◆; profile for maximum C_{max} = ■; and profile for minimum C_{max} = □. *Figure obtained from (Brailsford, Cowan et al. 2012)*

5.3.2 Data overview

5.3.2.1 Data quality (PCA)

At the beginning of the untargeted statistical analysis workflow, the data quality was inspected by the variation of QCs in the PCA score plot (**Figure 5-4**). The distribution of QC samples (N=17) was measured as 3.7 % and 4.4 % (at t[1] and t[2] directions, respectively) of the entire sample cohort range in the PCA plot, showing good overall reproducibility.

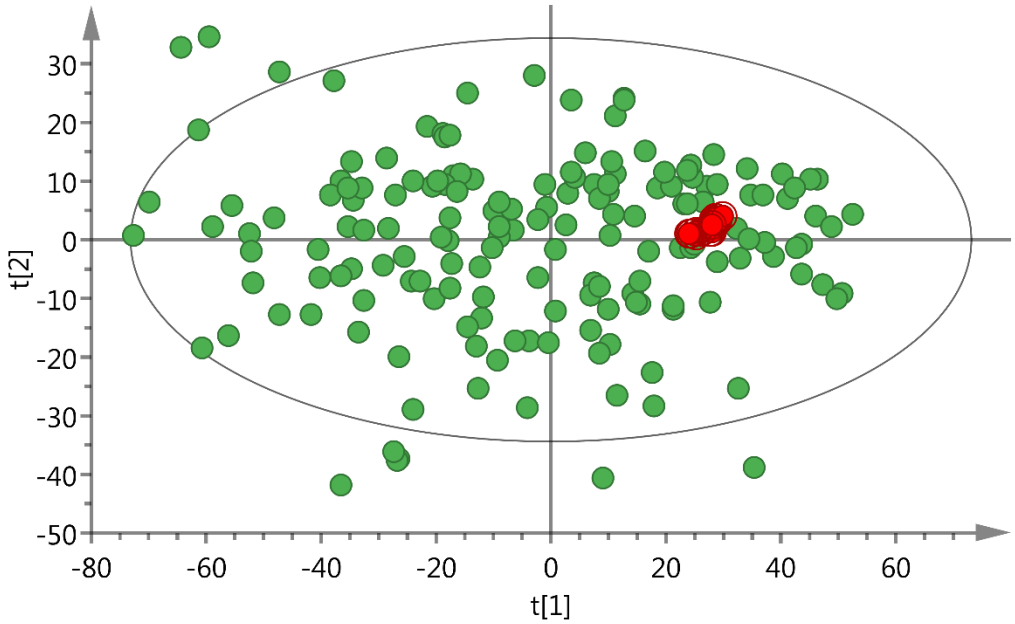

Figure 5-4 PCA score plot of all urine samples (N =168) and 17 QCs. Data were log-transformed and UV-scaled. The QCs cluster in same area of the PCA, suggesting across-run reproducibility.

5.3.2.2 PERMANOVA

Permutational multivariate analysis of variance (PERMANOVA) was conducted using the Euclidean distance. PERMANOVA is analogous to multivariate analysis of variance (MANOVA), which allows partitioning the variability of the data according to a complex design or model, and provides F-ratios that are analogous to Fisher's F-ratio in MANOVA (McArdle and Anderson 2001). PERMANOVA was used to detect possible significant differences in data between time and treatment (pre- and post-GHB administration), which were treated as fixed factors, and individuals as random factor. The PERMANOVA test result is shown in **Table 5-2**, which indicated significant change on treatment and along the time ($p < 0.005$ and $p < 0.001$ respectively).

Table 5-2 PERMANOVA test of the whole dataset

PERMANOVA[a] STRATA Individuals[b]					
	Df	Sums Of Sqs	Mean Sqs	F.Model	*p*
Treatment	1	3257	3257.3	3.65	0.002
Time[c]	1	7793	7793.2	8.73	<0.001
Residuals	150	133887	892.6	0.92	
Total	152	144937	1		

a. Data were log-transformed before PERMANOVA test.
b. Calculation excluded the individual difference among the volunteers.
c. Excluded time point 1, i.e. one week before GHB administration.

5.3.3 OPLS analysis

In order to search for the features with similar metabolism pattern as GHB, OPLS model was built using GHB as the Y-variable. The corresponding S-plot (**Figure 5-5**) indicated the features that the most positively correlated to GHB metabolism (top right in the S-plot), and 10 of them with largest p (corr) value were selected. However, most of the selected features showed random fluctuation along the time when univariate analysis was performed. That could be expected as there were significant GHB elevations only in 2 time points out of total 12 post-administration time points (see **Figure 5-3 and 5-6**), hence the model could be easily driven by the rest 10 time points. The feature at *m/z* 209.9830 showed a similar pattern with GHB (**Figure 5-6**), with significant correlation ($r = 0.517$, $p < 0.001$, Spearman's Correlation). Such correlation is an association and does not mean that the feature is indeed related to

GHB administration, however, if it were a metabolic change after GHB administration, it could help finding other metabolic paths for this drug. This feature was increased greatly at 24 and 30 hours which would have made it an ideal biomarker if it was causally related. However it was not possible to elucidate the structure of this small molecule and this feature remained unidentified. Additionally the lack of a direct control group (or diet control) in the experimental design of this clinical study meant that the biological origin of the feature was also unknown.

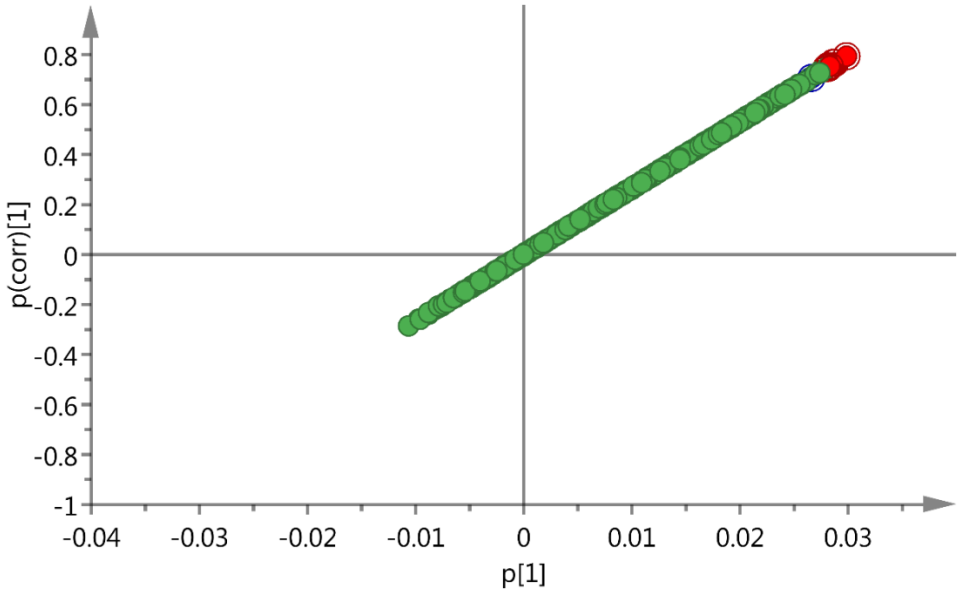

Figure 5-5 S-plot from OPLS model where GHB was set as Y-variable. The circles represent the features. X-axis represents covariate while y-axis represents correlation to GHB. The S-plot showed a straight line instead of "S" since the data were univariate-scaled. Features in the top right positively correlated to GHB, and 10 features with biggest p (corr) value were selected as features of interest and marked in red. Model fit: R2Y = 0.632, Q2 = 0.491.

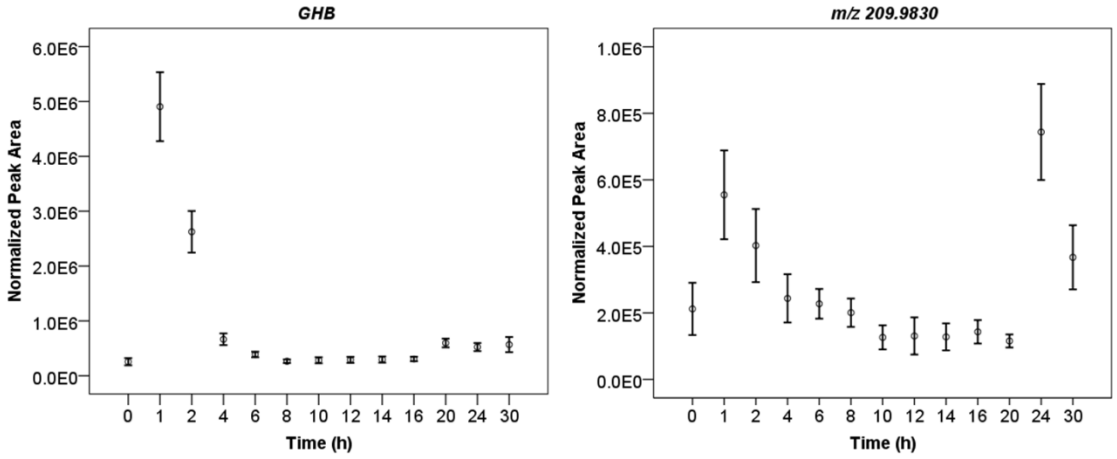

Figure 5-6 Concentration changes (Mean ± SEM) of GHB and feature at *m/z* 209.9830 during the pre- and 30 h post-administration. Left, GHB. Right, feature at *m/z* 209.9830. Y-axis represents the peak area normalized by total urine volume. Feature at *m/z* 209.9830 significantly correlated to GHB post-administration with r = 0.517, $p < 0.001$ (N=153, Spearman's Correlation).

5.3.4 PLS-DA analysis

To extract overall changes during the time, a PLS-DA plot covering all the time points was generated in R and the average position in each time point was calculated **(Figure 5-7)**.

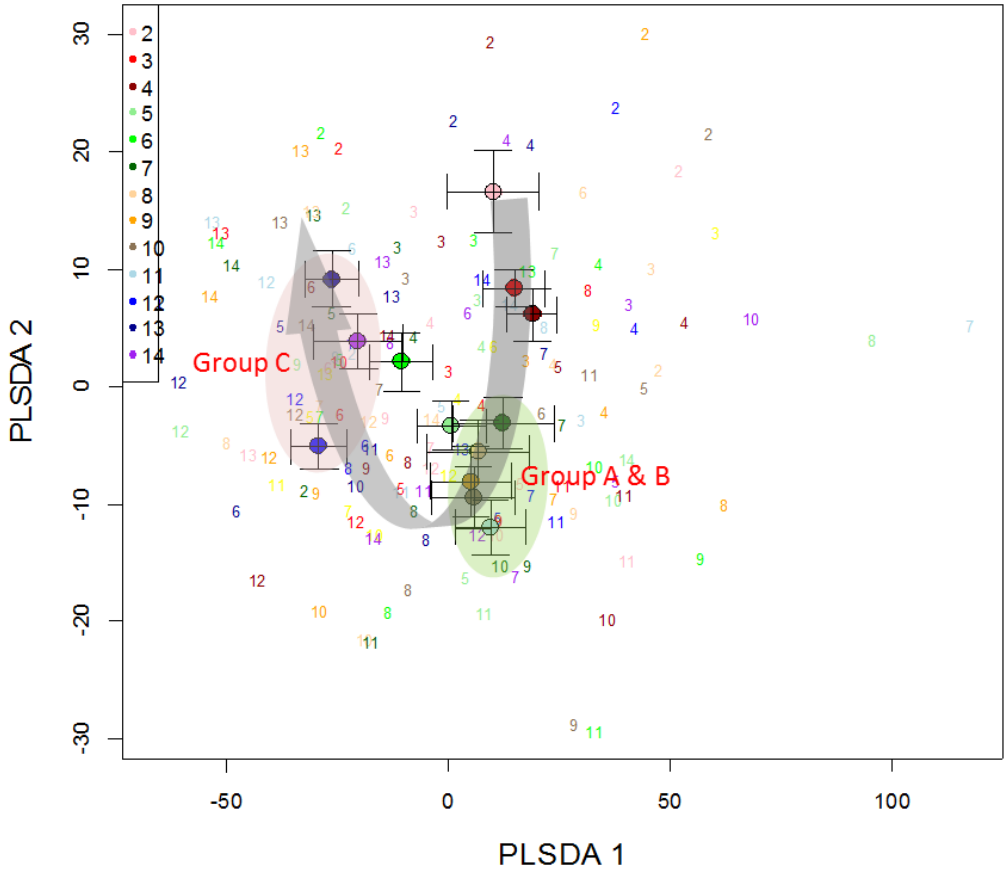

Figure 5-7 PLS-DA score plot showing a trend of the longitudinal moving. The numbers represent the time points, and each coloured circle represents the mean position (± SD) of the each time point. Group A and B: time points 7-11; Group C: time points 12-14.

PLS-DA using the whole "fingerprints" showed a trend of time points being driven away and then back towards the initial position. The time points were later divided into groups based on the clustering position to perform OPLS-DA analysis stepwise. It is also of practical use if biomarkers of different time periods can be found, as victims can go to test their urine in a wide range of time frame after being drugged. The group assignments using this information are shown in **Table 5-3**.

Table 5-3 Group assignments of stepwise analysis

	Group A	Group B	Group C
Time point No.	T7, T8, T9	T9, T10, T11	T12, T13, T14
Time (h)	8-12	12-16	20-30

5.3.5 Stepwise OPLS-DA analysis

Based on the group assignments in section **5.3.4**, stepwise analysis using OPLS-DA was performed between T2 (0 h) vs. Group A, T2 vs. Group B, and T2 vs. Group C, to find biomarkers of different time periods post-administration. **Figure 5-8** showed the OPLS-DA model of T2 vs. Group B. The score plot showed the separation of the two groups. To find the features of interest, initially 5 features in the S-plot with the highest p (corr) values were selected and then checked in the raw chromatograms to exclude noise features. Also, correlation tests were performed among those features provided they shared the same retention time. If the correlation coefficient was > 0.9, one or some of the features could be considered fragments or isotopes and excluded. Then another feature with the highest p (corr) value will be added in and checked as the procedure mentioned above, until a total of 5 features of interest were chosen. The concentration levels of those features were then plotted over the time from pre- to 30 h post-administration (**Figure 5-9**). The putative annotations and their possible origins are shown in **Table 5-4**.

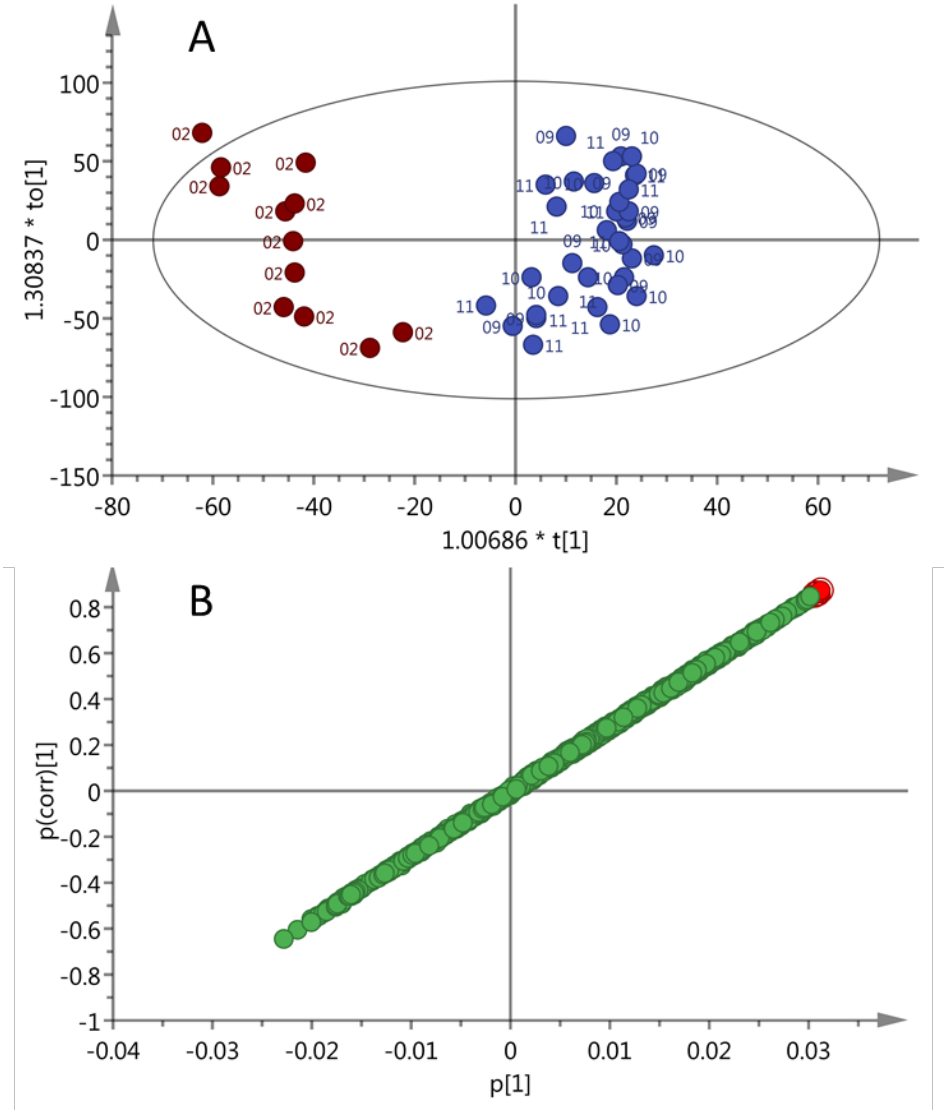

Figure 5-8 OPLS-DA model of T2 (pre-administration) vs Group B (T9, T10 and T11). A, OPLS-DA score plots in the predictive (x-axis) and orthogonal (y-axis) components of LC-MS data of urine samples, where dark red circles represent samples at T2, blue circle represent samples of Group B. B, Corresponding S-plot. Data include features from both positive and negative ionization modes, and were univariate-scaled and log-transformed. Model fit: R2Y = 0.894, Q2 = 0.675.

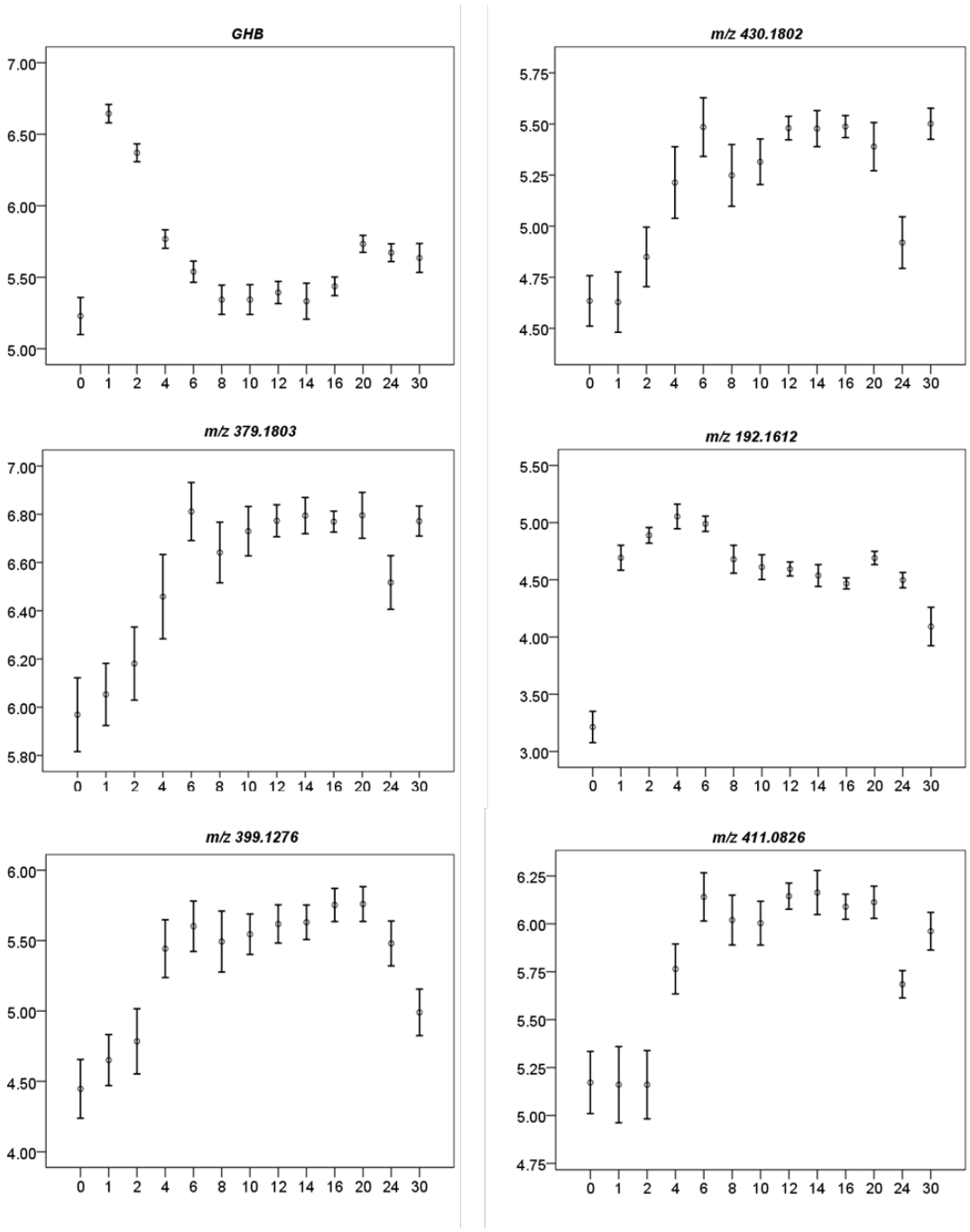

Figure 5-9 Concentration change of GHB in urine and the top 5 features selected via the S-plot of T2 vs Group B (T9, T10 and T11, 12- 16 h) during pre- and 30h post-administration. Data were presented in mean ± SEM. The x-axis represents time (h) and y-axis represents log-transformed normalized peak area.

Table 5-4 Annotated putative metabolites of top significant features in stepwise analysis. (Bold font indicates that the feature appears in more than one group)

Feature (*m/z*)	Putative metabolites	Origin
T2 vs. Group A		
192.1612 (+)[a]	***Unidentified***	_
277.0907 (+)	Biotin sulfone	Endogenous/Food
434.6143 (-)	*Unidentified*	_
414.1053 (-)	*Unidentified*	_
399.1276 (+)	**Riboflavin**	**Endogenous**
T2 vs. Group B		
430.1802 (+)	**Garcimangosone C**	**Food**
379.1803 (-)	Deacetylisovaltrate	Food
192.1612 (+)	***Unidentified***	_
399.1276 (+)	**Riboflavin**	**Endogenous**
411.0826 (-)	Patientoside A	Food
T2 vs. Group C		
192.1612 (+)	***Unidentified***	_
525.1493 (-)	Dihydroxychalcone	Food
430.1802 (+)	**Garcimangosone C**	**Food**
195.0285 (+)	*Unidentified*	_
392.0804 (-)	Isoorientin 2"-[feruloyl-(->6)-glucoside	Food

a. (+) : Feature detected in positive ionization mode.
(-) : Feature detected in negative ionization mode.

It can be observed in **Table 5-4** that the features at *m/z* 192.1612, *m/z* 399.1276 and *m/z* 430.1802 are the top discriminant features in more than one group, especially *m/z*

192.1612 which appears in the top list of all the 3 groups. However the only identified endogenous compounds are *m/z* 399.1276 as riboflavin and *m/z* 277.0907 as biotin sulfone, despite most of the features were putatively identified as compounds from food or remained as unidentified. The putative identifications were based on the rules mentioned in section **5.2.6**. For instance, *m/z* 192.1612 was first checked in databases, with no hits of +H and +Na adducts. There were several hits of $+NH_4$ adducts, nonetheless. After confirming the endogenous origin and reasonable log(p), the candidate molecules were then inspected in raw data for fragmentation. However, the fragments of these candidates were not found in the sample MS or MS/MS spectrum, in particular the molecular ions (without $+NH_4$). Thus m/z 192.1612 remains unidentified. Final confirmations with standards for putatively identified compounds are still needed, however more experiments with a control group will determine if a feature is worth confirming with standards.

5.3.6 Metabolic change between pre- and 24 h post-administration

In addition to the metabolic changes caused by GHB, circadian rhythm also plays a role in the urine metabolome changes (Giskeodegard, Davies *et al.* 2015). By comparing the profiles of pre-administration (0 h) and 24 h post-administration, and selecting features which do not return to basal concentrations in 24 h, the effect of features that follow circadian rhythm can be somewhat avoided. Features that increase greatly at 24h after GHB administration could be in turn related to GHB.

It is observed in **Figure 5-7** that the time points of pre- and 24 h post-administration do not overlap with each other, which indicates that the metabolic profile had changed

after a 24 h cycle. A volcano plot can be used to quickly identify the most meaningful changes in a large matrix (Cui and Churchill 2003). It plots significance (*p*-value) and fold-change (FC) on the y and x axes respectively. In **Figure 5-10**, the feature at *m/z* 192.1612 gives the highest fold-change (FC=12) and lowest *p*-value (p = 2.31E-08). This is the feature with the greatest change 24 hours after administration of GHB.

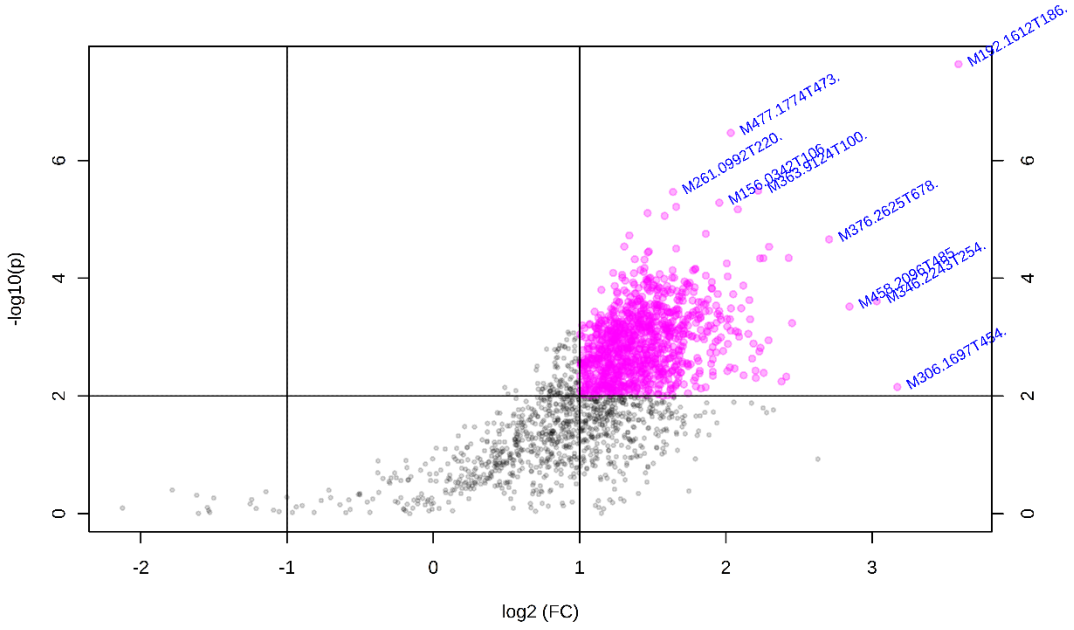

Figure 5-10 Volcano plot of pre- vs. 24h post-administration. X-axis represents the fold-change (FC) in $\log_2$ (FC), and y-axis represents the *p*-value in $-\log_{10}$ (p). The pink dots are the features with $p < 0.01$ and FC > 2, and it is observed that they were all upregulated after 24 h post-administration. The feature at *m/z* 192.1612 in the top right corner gives the highest fold-change of 12 and lowest *p*-value of 2.31E-08.

To test the capacity of the feature at *m/z* 192.1612 to predict the GHB administration at 24 h, Receiver Operating Characteristic (ROC) analysis was performed. The curve is created by plotting the true positive rate (TPR, also known as sensitivity) against the

false positive rate (FPR, also known as specificity) at various threshold settings, and it is able to test predictive properties on selected candidate. ROC curve gives an AUC of 0.986 for the feature at *m/z* 192.1612 (**Figure 5-11**), which is considered an excellent prediction for a potential marker. Yet our model might be over-fitted because the samples were not divided into training set and test set due to the limited sample size and further validation study is necessary to test biomarker capacity. The box plot of *m/z* 192.1612 showed significant concentration elevation at 24 h post-administration ($p < 0.001$, paired t-test). This feature also appeared in the top discriminant features list in stepwise analysis in each group (**Table 5-4**).

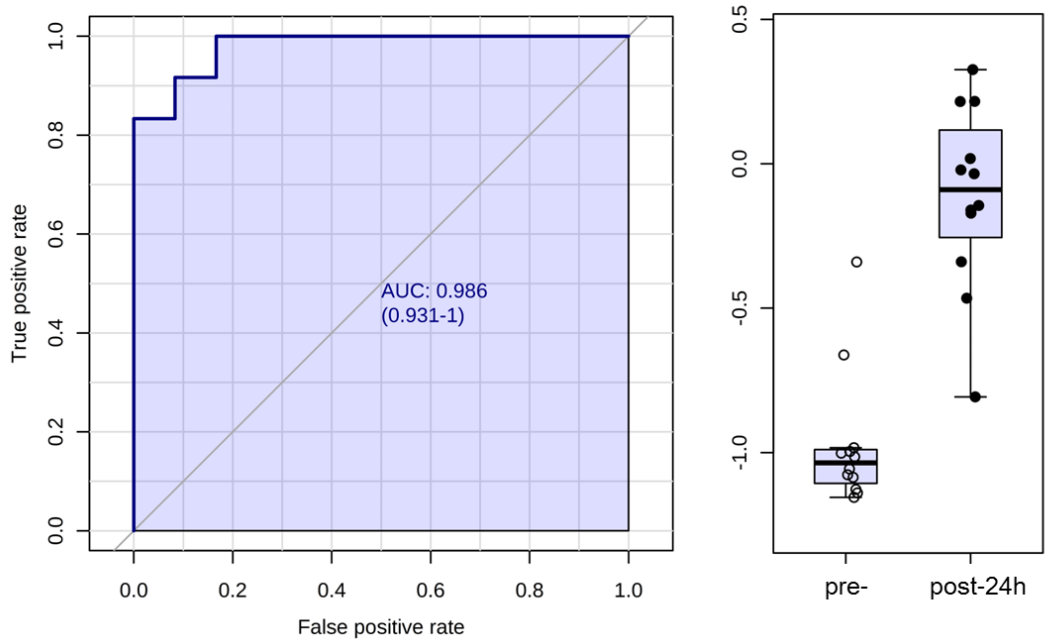

Figure 5-11 Receiver operating characteristic (ROC) curve (N=26) and box plots of the feature at *m/z* 192.1612. Data were univariate-scaled and log-transformed before analysis. The ROC curve returns an AUC of 0.986, 95% CI [0.931, 1]. The box plot for *m/z* 192.1612 in the pre- and 24 h post-administration groups shows the 75th and 25th percentiles and the median value. The whiskers represent the highest and lowest values that are not outliers. Outliers (values that are more than 1.5 the interquartile range) are represented by circles beyond the whiskers. The box plot demonstrated significant elevation of the feature at *m/z* 192.1612 at 24 h post-administration ($p < 0.001$, paired t-test).

Ideally, the major difference between pre- and 24 h post-administration should be driven by GHB and not by the circadian rhythm effects. However there was no control of the food intake for volunteers in this study, as a result the difference could be caused not only by GHB, but also by diet. This is likely the reason why some of the putative biomarkers in **Table 5-4** are originated from food. During an acute longitudinal monitoring period such as one or two days, the urine metabolic profile can be largely affected by food intake, thus the food control is essential. The possibility cannot be excluded that the feature at *m/z* 192.1612 and other features ($p < 0.01$ and FC > 2) in the volcano plot were originated from food provided that all the volunteers had similar food intake before or during the trial.

5.3.7 Limitations of this study

A limitation of this chapter is that linear mixed-effects models could have been a more appropriate statistical tool in longitudinal studies. However in this study, GHB was administrated in the same dose (25 mg/kg body weight) for all the volunteers. It is proposed that in a further study or other longitudinal study design, a multiple level of dosage could be given to build a linear mixed- effects model and visualize the predictive trend of potential markers.

As commented previously the biggest shortcoming of this study design was the lack of placebo group, i.e. control group of non-GHB administration. If there are samples taken from non-GHB administration group, the metabolic profile of each time point can be compared between treatment and control.

The environmental factors were not controlled and these could not be excluded. This is a big limitation of metabolomics in general as diet and xenobiotics will affect outcomes. The design could have taken into account biomarker specificity by employing volunteers from both GHB administrated group and non-administrated group with cross-over design, together with controlled food intake. These studies however demand a lot of resources, such as a metabolic facility where volunteers can be monitored continuously in-house.

5.4 Conclusions

In this chapter, a longitudinal phenotyping approach for discovering surrogate markers of administration of an endogenous compound GHB was developed. A total of 168 urine samples were analysed by UPLC®-QTOF MS and a total of 5,135 features were extracted by XCMS. QCs clustered in the centre of the PCA showing a good overall reproducibility of the experiment. PERMANOVA proved the metabolic profile change after GHB administration, and OPLS analysis found a feature at *m/z* 209.9830 significantly correlated to GHB. PLS-DA showed a trend of the time points longitudinally moving away and back towards the initial position in 30 h. Stepwise OPLS-DA analysis revealed the 5 most elevated features in each of the three different time period (8-12 h, 12-16 h and 20-30 h) post administration, in which riboflavin and biotin sulfone were putatively identified among the most discriminant features in 8-16 h and 8-12 h periods respectively. The unidentified feature at *m/z* 192.1612 showed significant changes in all the three periods. Further analysis of pre- and 24 h post-administration showed the feature at *m/z* 192.1612 having the largest fold change of

FC=12 and the smallest *p*-value of 2.31E-08. ROC curve of this feature returned a prediction capacity of 98.6%. These results suggest that the feature at *m/z* 192.1612 alone or together with other significant features found in stepwise analysis such as riboflavin and biotin sulfone, can be potential surrogate markers of GHB.

This preliminary research provides a general strategy for longitudinal metabolomics, it could be applied to a study which is food-intake controlled and a cross-over designed clinical trial. In the next chapter, a study design will be drafted taking into account some of the shortcomings, such as the lack of a control group, and an additional group will be added to exclude some random markers or false positives.

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Chapter 6 LC-MS based metabolomics discovers purine endogenous associations with low dose salbutamol in urine collected for anti-doping tests

6.1 Introduction

The importance and necessity of biomarkers in doping control have been demonstrated in Chapter 1 and Chapter 5. In short, biomarkers have the potential to facilitate the discovery of new doping agents with biological activities similar to related named prohibited substances, as well as improving the sensitivity and prolonging the detection time window.

The identification of a metabolomic biomarker requires access to samples obtained from individuals who have administered the substance under investigation. This could be challenging when dealing with sport samples. Indeed, sport samples can be utilised for research only if the appropriate research consent has been agreed by the athlete and only after the required storage period of three months has expired. Further to this, samples that generated adverse analytical (positive) findings must be stored sealed for several years in case further analyses are requested. This makes positive samples not readily accessible for research purposes.

On the other side, according to WADA regulations, the presence of "threshold" substances (such as salbutamol, ephedrines, morphine and a few others) in urine constitutes a doping violation only if their concentration is determined to be above a certain threshold (WADA 2014). Samples that show the presence of these substances below the threshold are negative for the purpose of doping control, but represent an easily accessible surrogate of "positive" samples for research purposes.

Following these considerations, we selected salbutamol as the model exogenous compound, based also on the availability and accessibility of samples in our laboratory. Salbutamol (albuterol), [2-(tert-butylamino)-1-(4-hydroxy-3-hydroxy methylphenyl) ethanol] (**Figure 6-1**), is a relatively selective β2-adrenergic bronchodilator commonly used as an aerosol for relieving the acute symptoms of asthma (Zhang, Teng *et al.* 2012). The list of prohibited substances in sport published by WADA specifies that the use of salbutamol is only permitted by inhalation of therapeutic doses (WADA 2016). Administration by oral or parenteral routes and administration of very large inhaled doses (greater than 1,600 μg in any 24 hours) are prohibited due to a strong adrenergic stimulatory effect and anabolic-like effect (Martineau, Horan *et al.* 1992, Caruso, Signorile *et al.* 1995). By contrast, administration of therapeutic inhaled doses has no observable ergogenic effect (Meeuwisse, McKenzie *et al.* 1992). A urinary concentration of salbutamol in excess of 1,000 ng/mL is considered a doping violation, unless an appropriate pharmacokinetic study can prove that this is the result of abnormal metabolism when the compound is taken according to the permitted administration. The threshold concentration is based on the sum of the glucuronide conjugate and free drug concentrations according to currently effective WADA technical documents (WADA 2014). In a previous study, salbutamol glucuronide was found in urine at less than 3% compared with unconjugated salbutamol after oral administration and less than 1% in authentic doping control urine samples (Mareck, Guddat *et al.* 2011). For this reason, this study focused on salbutamol only in its free form.

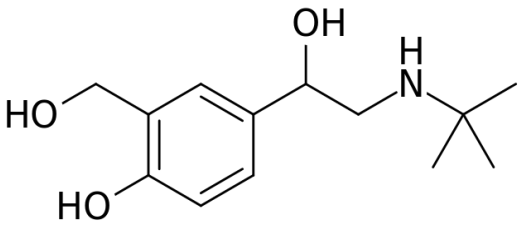

Figure 6-1 The molecular structure of salbutamol.

Several studies have used metabolomics to find the biochemical paths implicated with doping substances. Boccard *et al*. discovered several metabolites relevant to the detection of testosterone intake, and suggest both glucuronide and sulphate steroid conjugates can be include in the Athlete Biological Passport (ABP) (Boccard, Badoud *et al.* 2011); Raro *et al.* also found 1-cyclopentenoylglycine as a potential marker for testosterone ester misuse (Raro, Ibanez *et al.* 2015); for salbutamol Kiss *et al.* found increased pantothenic acid (vitamin B5) in a salbutamol positive group (Kiss, Lucio *et al.* 2013).

Detection of salbutamol is not particularly challenging, but this substance can be used as the model compound to demonstrate the feasibility of an approach that can later be extended to a much larger cohort of substances.

The main purpose of this chapter is to find surrogate markers induced by the intake of small amounts of salbutamol and explore a sensitive approach to screen biomarkers of drug misuse.

6.2 Methods

6.2.1 Chemicals and materials

All solvents used were HPLC grade. Acetonitrile and methanol were obtained from Fisher Scientific (Loughborough, UK). Formic acid (99–100 %) was purchased from VWR (Leicestershire, UK). Ultrapure water (18 MΩ · cm) was obtained from an ultra-pure water system (Elga, UK).

6.2.2 Urine samples

The samples from antidoping tests were acquired after professional sport competition and came from diverse demographics at random times of the day. A total of 91 urine samples were obtained from 91 different athletes, who had indicated their consent for research on their samples, with 30 salbutamol detectable samples (DET, 10 - 1000 ng/mL,19 males and 11 females) and 61 salbutamol non-detectable samples (NON-DET, less than 10 ng/mL, 40 males and 21 females). Samples were obtained from a total of 18 sports, 11 of which were classified as endurance sports. The number of samples for endurance sports were matched in both groups: the DET and NON-DET groups. The Specific gravity was measured by a calibrated DM40 densitometer (Mettler-Toledo International, Switzerland). All urine samples were aliquoted and stored in polypropylene tubes at -20 °C for at least three months and anonymised prior to analysis to comply with the ethical requirements of the World Anti-Doping Agency.

6.2.3 Sample preparation

To make a spiked (SPI) group, 30 independent samples (19 male and 11 female) from NONDET were spiked with salbutamol to final concentrations at 1, 2, 5, 10, 20, 50,

100, 200, 500 and 1000 ng/mL. A pooled sample from 20 urine samples with an equal number of DET and NON-DET was used as a quality control (QC). All the urine samples were centrifuged at 11,000 rpm for 15 minutes to remove any particles. An aliquot of 50 µL of supernatant was diluted with 200 µL ultrapure water previously spiked with internal standard mix (IS) containing mefruside and D3-salbutamol both at 500 ng/mL. The solution obtained was pipetted into amber HPLC vials with inserts. Additional samples containing pure hypoxanthine standard obtained from Sigma-Aldrich (St. Louis, MO, USA) were prepared for compound identification.

6.2.4 UHPLC–HRMS analysis

For a detailed description of chromatographic separation and mass spectrometer methods, refer to section **4.2.5**.

6.2.5 Data processing and analysis

For a detailed description of data conversion and XCMS settings, refer to section **3.2.5**.

The data from both positive and negative ionization mode were then normalized by the specific gravity of each sample, before importing into SIMCA (UMetrics, Umeå, Sweden), where it was subjected to multivariate data modelling, including pareto-scaled principal component analysis (PCA) (Figure not shown) and log-transformed orthogonal partial least-squares discriminant analysis (OPLS-DA) with corresponding S-Plot analysis. The features with p(corr) >0.25 or <0.45 were selected and re-evaluated using OPLS-DA again. The OPLS-DA model was cross validated 7-fold and a *p*-value was calculated. Graphical outputs were prepared in SIMCA.

To test predictive properties, the receiver operating characteristic (ROC) was used. ROC curves with area under the curve (AUC) statistic and 95 % confidence intervals (CI) for salbutamol, a chosen feature and creatinine were recorded. Mann–Whitney *U* test was used on the features in DET and NON-DET and Spearman's correlation were calculated between selected features and salbutamol. Box and whisker plot to demonstrate the data distribution were calculated using raw data measured as area under the peak and then normalized to IS and specific gravity of the feature was used. Analyses were performed using SPSS (IBM SPSS statistics, version 22). Features were investigated for fragmentation, *m/z* and retention time for identification purposes and Post-hoc analysis for statistical power using G*power (http://www.gpower.hhu.de/) was conducted (two tails, α=0.05, Normal distribution was assumed). **Figure 6-2** illustrates the general experimental overview.

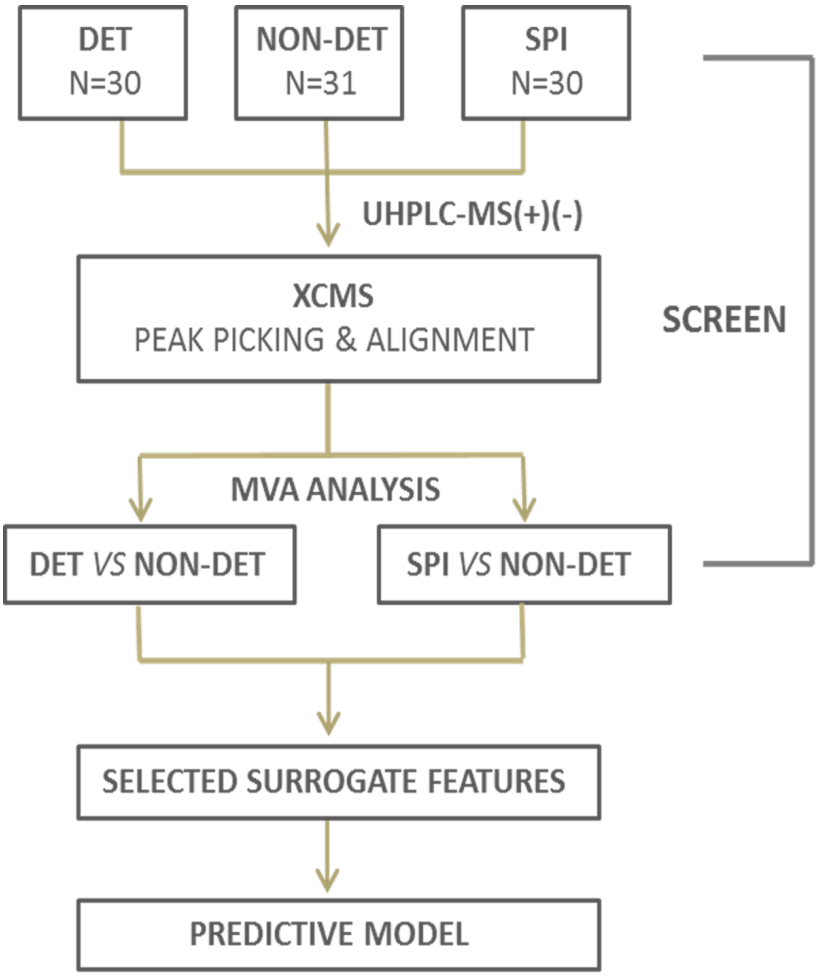

Figure 6-2 Experiments overview. (DET, salbutamol detectable samples; NON-DET, salbutamol non-detectable samples; SPI, NON-DET samples spiked with salbutamol reference standard). n= 91 samples were injected into UHPLC-MS in polarity-switching mode, data were processed in XCMS before multivariate analysis: DET against NON-DET and SPI against NON-DET samples.

6.3 Results and discussion

6.3.1 Normalisation method

As mentioned in Chapter 3, specific gravity is the standard normalization method in doping control recommended by WADA, hence specific gravity was applied as the normalization method for this metabolomic study on salbutamol.

6.3.2 Surrogate marker screening

A total of 3895 (+, acquired in the positive mode) and 3159 (-, acquired in the negative mode) distinct features were observed. PCA modelling showed a repeat extraction of a urine pool indicating the good reproducibility (figure not shown). OPLS-DA modelling demonstrated DET isolation from NON-DET. The corresponding correlation S-Plot was used to generate a list of features of interest that were of importance to the discriminatory model and increased in DET, which could contain candidate surrogate markers. To enhance the specificity of the model, in the initial S-Plot of salbutamol DET versus NONDET (figure not shown), features with p (corr) >0.25 or <-0.45 were selected to build another OPLS-DA model (**Figure 6-3A**), with a *p*-value = $6.8e^{-7}$. **Figure 6-3** illustrates the models using data from the positive ionization mode**.** **Figure 6-3A** showed that the two groups can be clearly separated. The same protocol was applied to SPI vs. NON-DET model. In the two models, surrogate markers account for the difference between DET and NON-DET in addition to salbutamol, while the only difference between NON-DET and SPI is salbutamol.

However, as confounding effects due to the analytical workflow and the diversity and individual differences of human urine occur, a third group was created to assess for "random markers". Thus, a group of samples spiked with salbutamol (SPI) was set to eliminate possible confounding effects. Two S-Plots from DET versus NON-DET and SPI versus NON-DET groups can help directly visualize the result (**Figure6-3B and 6-2C**). Features with p (corr) >0.5 in **Figure 6-3B** were highly correlated to salbutamol and were considered features of interest. The model in **Figure 6-3C** was constructed with urine samples that were controls spiked with salbutamol (SPI) versus control urine (NON-DET), in this figure the features that are highly correlated to SPI group were regarded as false positives. Feature at *m/z* 381.0792 seen in NON-DET versus DET (crossed square in **Figure 6-3B**) was considered a false positive as it was also mined **in Figure 6-3C** and correlated to the salbutamol spiked group. It was hence considered as not associated to salbutamol metabolism and excluded from further consideration. The same protocol was applied to the negative ionization data, the figure for the negative ionization OPLS-DA was added in the supplementary information **Figure S6-6**.

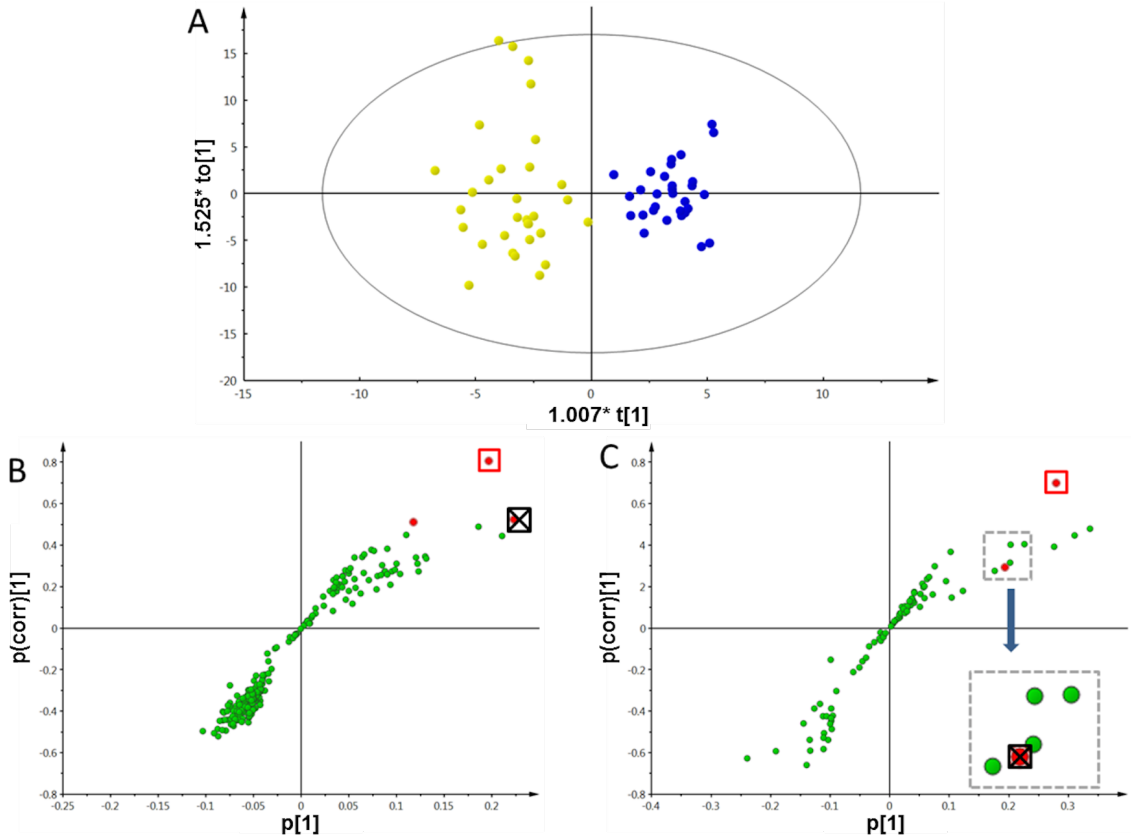

Figure 6-3 OPLS-DA Models in positive ionization mode. A, Score plot of the OPLS-DA model from DET vs. NOT-DET. Blue, DET; Yellow, NON-DET. B, S-Plot from A. C, S-Plot of an OPLS-DA model from SPI vs. NON-DET. Features of interest were marked in red in B. Salbutamol, solid red boxed; a "false positive" marker, *m/z* at 381.0792, solid black boxed and crossed.

6.3.3 Exploration results

Two features of interest with distinct mass and retention time from both ionization modes were selected after the procedures mentioned above. The feature at *m/z* 137.0458 (in positive ionization mode) was identified as hypoxanthine against a pure reference standard (**Figure 6-4**). The feature at *m/z* 336.1134 (in negative ionization mode) remained as unidentified.

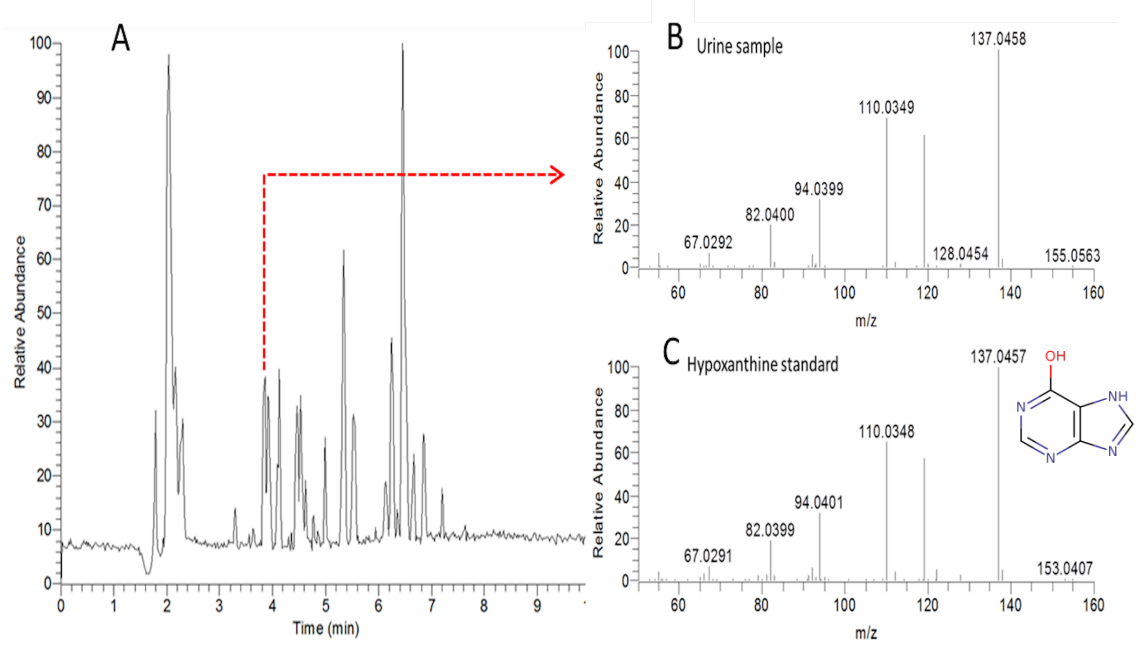

Figure 6-4 A chromatogram of a urine sample and the MS spectra of hypoxanthine. A, An example chromatogram of a typical urine sample. B and C, High resolution tandem MS/MS spectra of the feature at *m/z* 137.0458 at retention time 3.80 min obtained from and a urine sample (B) and hypoxanthine reference standard (C). Mass accuracy< 3ppm.

A ROC curve for hypoxanthine, salbutamol and creatinine from the fingerprinting data was performed. The curve is created by plotting the true positive rate (TPR, also known as sensitivity) against the false positive rate (FPR, also known as specificity) at various threshold settings, and it is able to test predictive properties of selected candidate. Salbutamol was chosen as positive reference and creatinine is a waste product of muscle-building processes and reflects muscle turn-over, the latter was used as a reference to compare the specificity of hypoxanthine and salbutamol. Salbutamol, hypoxanthine and creatinine gave the area under the curve of 0.983 (p <0.001), 0.790 (p <0.001) and 0.542 respectively (**Figure 6-5, Table 6-1**). The accuracy, sensitivity

and specificity of each compound are shown in **Table 6-1**. Samples in SPI contain salbutamol but are negative for surrogate markers, thus these independent samples were combined with the NON-DET group in ROC test for hypoxanthine and creatinine (n=91). Only DET and NON-DET groups were used for ROC test of salbutamol (n=61).

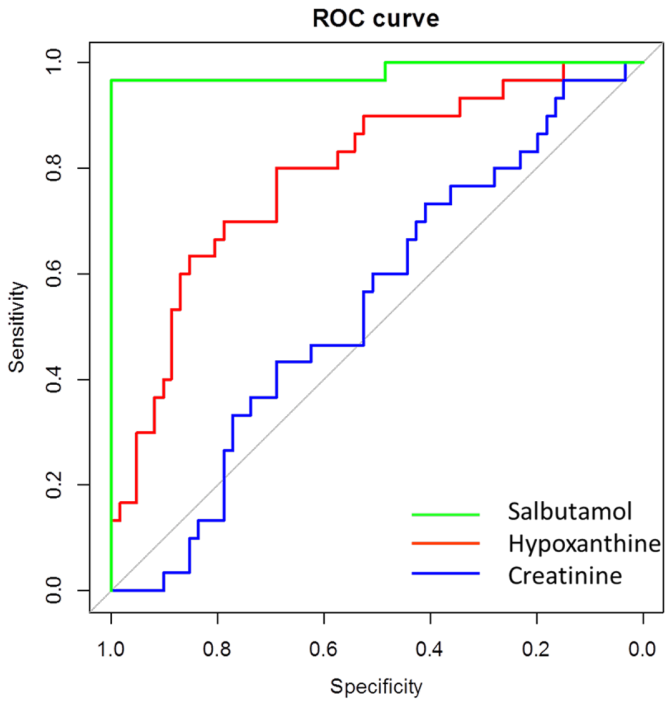

Figure 6-5 Receiver operating characteristic (ROC) curve of salbutamol (n=61), hypoxanthine (n=91) and creatinine (n = 91).

Table 6-1 Descriptive parameters for the ROC analysis

	AUC	Accuracy	Sensitivity	Specificity
Salbutamol[a]	0.983	0.984	0.967	0.967
Hypoxanthine[b]	0.790	0.769	0.633	0.836
Creatinine[b]	0.542	0.670	0.900	0.180

a, Calculations were based on 61 samples.
b, Calculations were based on 91 samples.

The ROC curve of hypoxanthine suggested a predictive potential of near 80%, which is currently regarded as a minimum requirement for biomarkers (Report 1998, Whiley, Sen *et al.* 2014). In the meanwhile, creatinine showed a predictive potential of 54%, which was considered as poor predictor. This proved hypoxanthine is more "salbutamol-specific". Yet our model might be over-fitted because the samples were not divided into training set and test set due to the limited sample size and further validation study is necessary to test biomarker capacity. Univariate analysis after semi-quantitation showed a significant increase of hypoxanthine in DET (**Figure 6-6**). Post-hoc analysis yielded a statistical power of 90 % for the effect size of hypoxanthine in this study. Spearman's correlation showed the correlation coefficient of hypoxanthine to salbutamol to be 0.415, $p < 0.01$.

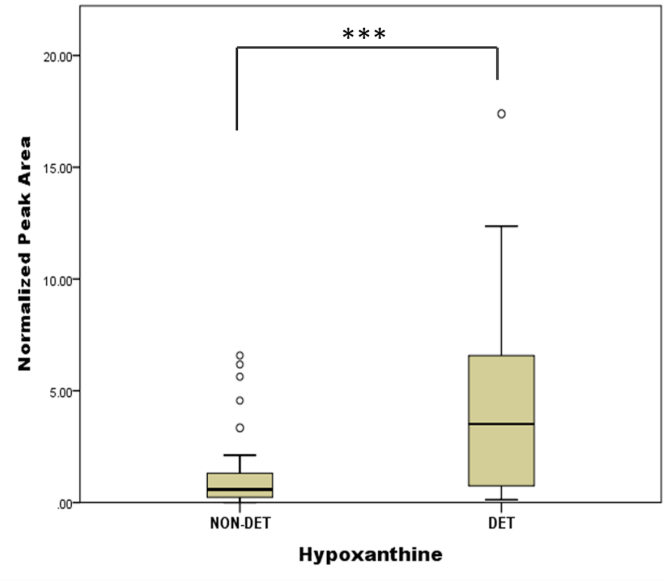

Figure 6-6 The box plot for hypoxanthine in the DET and NON-DET groups shows the 75th and 25th percentiles and the median value. The whiskers represent the highest and lowest values that are not outliers. Outliers (values that are more than 1.5 the interquartile range) are represented by circles beyond the whiskers. *** p <0.001, Mann–Whitney U test.

The metabolite identified here as being correlated with salbutamol is an energy-derived metabolite that indicates high (adenosine triphosphate) ATP turnover (Hellsten, Sjodin *et al.* 1998, Stathis, Zhao *et al.* 1999). Hypoxanthine is a purine derivative and it is one of the end points of purine metabolism in humans. The level of hypoxanthine in plasma is a marker of adenine nucleotide metabolism **(Figure 6-7)** and its rise in plasma reflects greater efflux from the muscle following exercise (Sahlin, Tonkonogi *et al.* 1999). Urinary hypoxanthine excretion indirectly represents a net loss of ATP from the muscle, chiefly because of the metabolic stress of intense activity (Stathis, Zhao *et al.* 1999, Gerber, Borg *et al.* 2014). Hence in our study, the higher concentration of hypoxanthine in DET may result from higher ATP consumption.

The next step was to investigate hypoxanthine up- and down-stream metabolites. A strong correlation was observed with its direct downstream metabolite xanthine (Spearman´s correlation 0.706, $p < 0.001$). Similar yet weaker correlations to hypoxantine were observed for the purine metabolites: urate, inosine and methyladenosine. Interestingly, xanthine also showed significant correlation with salbutamol (Spearman´s correlation 0.358, $p < 0.01$) and had a p (corr) value in S-plots in OPLS-DA model just under our threshold p (corr) > 0.4. **Table S6-6** in Supplementary Information shows these data.

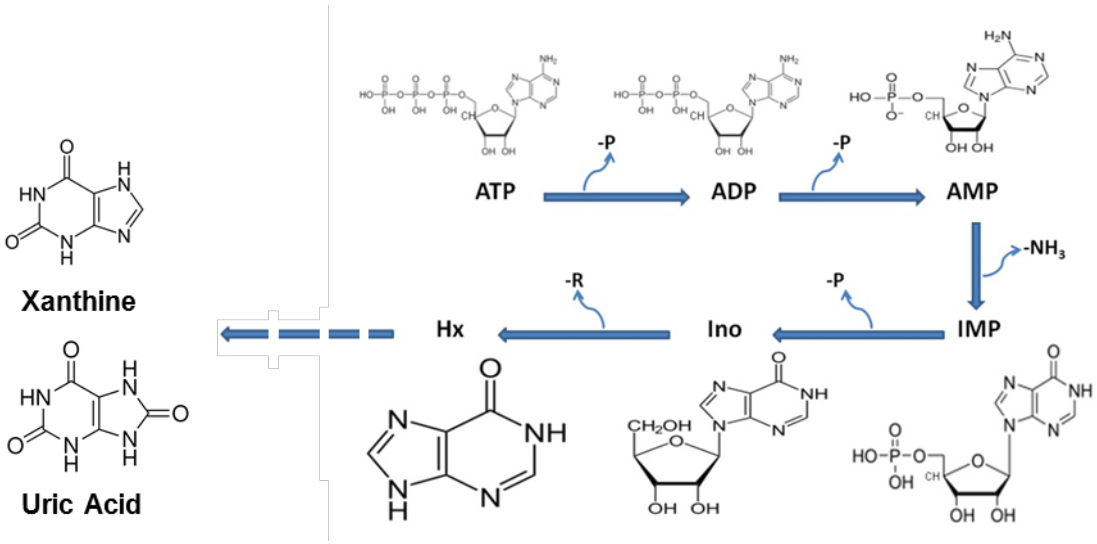

Figure 6-7 A general scheme with molecular structures of ATP depletion pathway to Hypoxanthine (Hx) and finally Xanthine and Uric acid.

Interestingly, Zieliński *et al.* found that hypoxanthine was a strong indicator of performance in highly trained athletes and its prediction ability was quite good regardless of sport disciplines (Zielinski, Krasinska *et al.* 2013). As stated above, salbutamol is a drug that can improve respiratory adaptation (Goubault, Perault *et al.* 2001) and enhances sport performance in large doses. Whilst the sport disciplines of

the athletes were well mixed in each group in this study to exclude the effect the endurance sports may cause, our finding provides a possible link between endogenous compounds and salbutamol, indicating the combined detection of salbutamol and some endogenous molecules. The follow up to this pilot study is to understand hypoxanthine increase in relation to the administration of salbutamol. To this end experiments using controlled athletes will be performed to study if salbutamol administration has a measurable effect on hypoxanthine in urine. Indeed, though the samples in the DET group contained salbutamol below the WADA threshold, we cannot exclude the scenario where these athletes had been taken larger doses of salbutamol for a long period and reduced or stopped the dose before being tested.

6.4 Conclusions

A small molecule phenotyping approach for discovering a surrogate marker of salbutamol administration was developed. A total of 91 sport urine samples were analysed by UHPLC-Q Exactive MS. Three groups (DET, NON-DET and SPI) were created and two models were built. Hypoxanthine was identified as a potential surrogate marker of salbutamol, with the ROC curve returning a positive actual state prediction of 79 %, and showed a significantly increased trend in the salbutamol-containing group, but not in the negative and spiked groups.

This metabolomics approach to screen biomarkers of drug misuse should be applied to a larger cohort of doping substances in controlled longitudinal experiments. Endogenous metabolites such as hypoxanthine could aid the preliminary screening of

urine samples that tested negative using conventional screening methods. This strategy could be used as an intelligence-based complementary approach for targeting of athletes for further testing.

6.5 References

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6.6 Supplementary Information

Supplementary figure and table

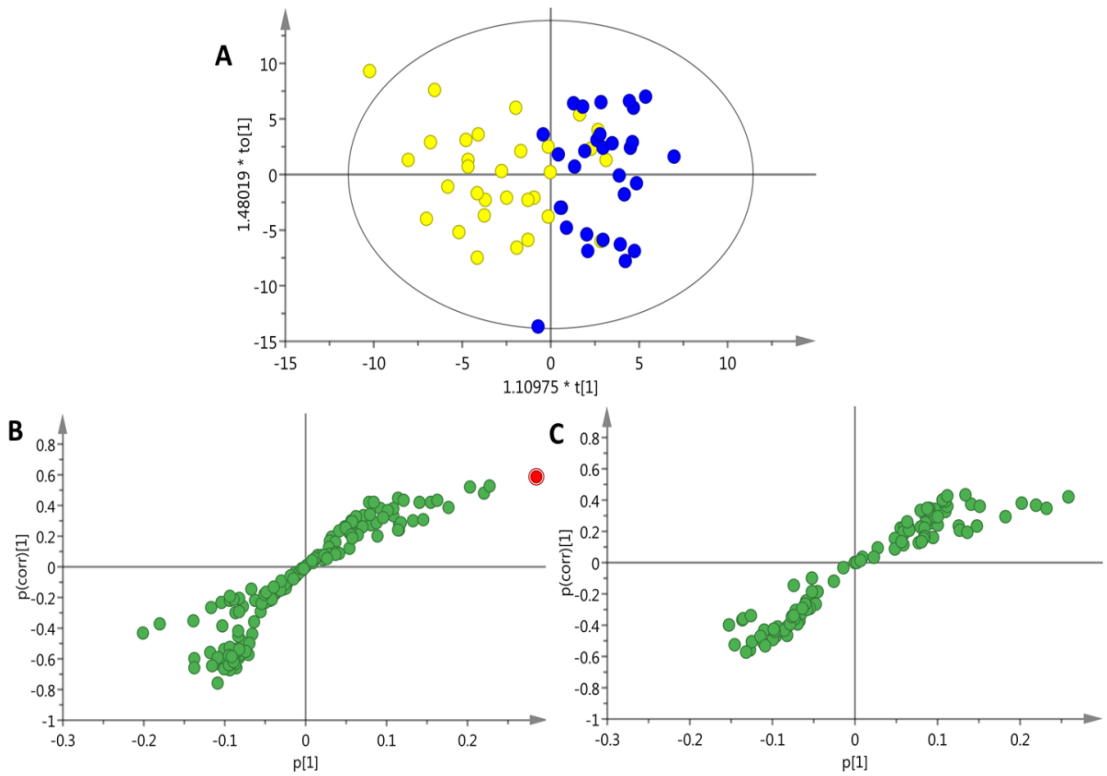

Figure S6-8 Models in negative ionization mode. A, OPLS-DA model. Blue, DET; Yellow, NON-DET. B, S-Plot from A. C, S-Plot of an OPLS-DA model from SPI vs. NON-DET. Feature of interest was marked in red, and it was not appeared in C. The feature *m/z* 336.1134 in negative ionization mode was most correlated to DET group and was selected as feature of interest, but it could not be identified.

Table S6-2 Correlation coefficients for purine metabolites with salbutamol (SB) and hypoxanthine (Hx)

	p(corr) in S-plot of M1[a]	p(corr) in S-plot of M2[b]	Spearman´s correlation with SB[c]	Spearman´s correlation with Hx[d]
Salbutamol	0.588	0.806	NA	NA
Hypoxanthine	0.629	0.511	0.415***	NA
Xanthine	0.382	0.253	0.358**	0.706***
Uric acid	-0.220	NA	0.303*	0.300**
Inosine	-0.002	NA	-0.062	0.252*
N1-Methyladenosine	0.112	NA	-0.113	0.282**

a, M1, the initial OPLS-DA model
b, M2, the OPLS-DA model built on selected features with p(corr) > 0.25 or < -0.45 in M1
c, Calculations were based on 61 samples.
d, Calculations were based on 91 samples.
*** $p<0.001$, ** $p<0.01$, * $p<0.05$

Chapter 7 Final conclusions and future directions

7.1 Final conclusions

Traditional anti-doping analytical methods in sport are mainly tailored to the targeting of known drugs and endogenous molecules, which cause difficulties in rapidly reacting to emerging threats, such as designer drugs, biological therapeutic agents and new technologies. These threats require radically different approaches, among which the use of metabolomics to find biomarkers is considered a promising strategy.

To this end a number of studies were designed and undertaken, and the results were presented and discussed in the previous chapters of this thesis.

In chapter 2, the study aimed at improving the amount of information obtained by using untargeted analysis in dried spots, which at the same time show advantages in sample collection, transport and storage. In-vial extraction (IVE), in-vial dual extraction (IVDE) and UHPLC-MS analysis were employed for metabolic fingerprinting. Firstly, an analytical method for dried biofluid spot metabolomics was designed and tested using samples from rat serum and RPMI cell nutrient medium. Univariate analysis of selected metabolites and multivariate analysis of the whole dataset showed acceptable reproducibility for the method. Secondly, the application of the IVDE method to different dried biofluid spots from rats and mice resulted in reproducible metabolic fingerprints, with sufficient feature coverage for high-throughput metabolomics experiments. The small sample sizes (10-15 μL) required for the spots, and the low transfer losses associated with the IVDE method, suggested that the spot-IVDE procedure could be adopted for fingerprinting and metabolomics

studies. However the possibility of loss of sensitivity due to the spotting and extraction procedure might be a risk that should be tested versus liquid samples.

In Chapter 3, a protocol for the UHPLC-Q-Exactive™ Orbitrap-MS was designed in order to obtain high mass accuracy in non-targeted metabolomics. One of the bottlenecks of metabolomics is identification of metabolites and high mass resolution can greatly improve it. The study was based on finding the best instrument resolution and producing a data mining workflow. By using conventional liquid samples, the results showed that fingerprinting using this protocol measured more than 3K features in urine at the resolution of 70,000 FWHM, giving more features compared to the resolutions of 140,000 and 17,500 FWHM. Multivariate analysis also revealed that the largest number of discriminant features (96) was obtained at the resolution of 70,000. Therefore it was initially chosen as the MS resolution in our protocol and PCA plot showed a clear separation in a pilot study using 91 real urine samples and 10 QCs. Multivariate PCA analysis showed overall analytical stability of the protocol across the 101 samples. Hence the protocol was deemed of good quality and ready for practical applications with further analysis of data interpretation.

Chapter 4 brought the work performed in chapters 2 and 3 together, by evaluating IVE of dried urine spot metabolomics and comparing it with conventional urine liquid sampling. Additionally spots were assessed for one-week freeze-thaw stability in terms of a data quality test, feature numbers and multivariate analysis. The protocol developed in chapter 2 was applied and results showed IVE paper-spot sampling can indeed cause some information loss but proved to be robust and similar to

conventional sampling when used for urine metabolomics. Paper spots also showed good reproducibility after a one-week freeze-thaw circle. All of these suggested paper spots could be a good alternative when sample volumes are limited, such as in studies when many "omic" analyses are required from one sample.

In chapter 5, the metabolomics strategy was applied to the samples remaining from a clinical trial, hence applying the metabolomics approach for discovering surrogate markers of the administration of an endogenous compound, GHB. The focus was to find metabolites that were related to GHB and had a longer detection time than GHB. A total of 168 urine samples were analysed by UPLC®-QTOF-MS. A battery of multivariate statistical tools was chosen to mine the data and understand the effects of GHB with time in the urine fingerprints. The results suggested that the feature at *m/z* 192.1612 alone or together with other significant features found in stepwise analysis such as riboflavin and biotin sulfone, might be potential surrogate markers of GHB. These preliminary results provide a general strategy for longitudinal metabolomics despite several shortcomings in the study design. Findings were limited by the lack of a placebo and diet control. In the future the statistical workflow could be applied to a study which is food-intake controlled and cross-over designed, in this way maximizing the chance of discovering biomarkers.

In chapter 6, a metabolomics approach for discovering a surrogate marker of salbutamol, an exogenous doping substance, was attempted. A total of 91 sport urine samples were analysed by UHPLC-Q Exactive MS. Three groups (positive, negative and spiked) were created and two models were built. Hypoxanthine was identified as a

potential surrogate marker of salbutamol use, with the ROC curve returning a positive "doping" prediction of 79 %, and showed a significantly increased trend in the salbutamol-containing group, but not in the negative and spiked groups. These findings need further validation but meanwhile the strategy could be used as an intelligence-based complementary approach for targeting of athletes for further testing.

In summary, Chapter 2, 3 and 4 supported the general hypothesis in methodological aspect, addressing the practical possibilities of HRMS for small sample volume fingerprinting. In chapter 5 and 6, the metabolomics strategy was applied to actual doping samples to validate the hypothesis. This thesis has shown that, following the protocols and strategies developed herein, LC-MS based metabolomics can provide novel information in the doping field and that metabolomics, as an untargeted discovery tool, can contribute in addressing emerging threats in sports. Endogenous metabolite alterations such as hypoxanthine could aid the preliminary screening of urine samples that tested negative using conventional screening methods.

7.2 Future directions

Further validation of hypoxanthine as a marker and biological interpretation

Hypoxanthine was found as a novel potential biomarker of salbutamol in the last chapter using samples from anti-doping tests. To be accepted and utilized in anti-doping tests, the markers should be further validated and it would ideally be specific to salbutamol or the same category of β2-agonist. Therefore the primary interest for the future work is to validate this potential marker in a controlled longitudinal study, with

salbutamol administrated in different doses. By comparing the urine and plasma concentrations of hypoxanthine with different single dosing and multiple dosing of salbutamol administration, it is hoped that a direct or clearer relationship could be established between the two compounds.

As an example, alternative future work direction would be to investigate the actual biochemical pathways and imbalances behind the concentration variations of hypoxanthine and other purine metabolites. For this, other different analytical techniques can be introduced to better detect the up- and down- stream metabolites of hypoxanthine such as using HILIC and measuring AMP and ATP. An approach such as this could identify novel pathway targets of salbutamol or β2-agonist and help improve the understanding of the mechanism of β2-agonist, leading to novel detection targets.

Applications in other doping compounds or technologies

Another inspiring further work pathway would be to apply the protocol established in this thesis to investigate some untraceable doping compounds or technologies such as hGH, EPO and autologous blood doping. Although there have already been some preliminary findings on the biomarker of these doping substances and technologies, metabolomics can still provide complementary paths and possibly more biomarkers to be added in the Athlete Biological Passport (ABP) to enhance the specificity.

Moreover, in the future, substances that are pathway related could be grouped and the same category investigated together to find the common biomarkers, which would be

of great value to help screen for the so-called "designer drugs". By monitoring the biomarker as a function enhancer group, the use of a designer drug with minor structure modifications could be possibly pinpointed assuming similar biological activities achieved with known substances.

Sample sharing system across anti-doping laboratories using paper spots

Although a paper-spot metabolomic method was developed and validated in this thesis, it has not been applied in anti-doping practice yet. For the last two chapters of anti-doping applications, paper-spot method was not used because we had access to the neat urine samples in the same building, and we wanted to acquire as much information as possible. However, it is indeed difficult to find a large number of urine samples positive to one drug to carry out a metabolomic study in one WADA accredited laboratory. Since positive urine samples will be stored for a long period, it would be of significant value to make use of these samples first instead of expensive clinical trials and complicated approval procedures. Herein it is proposed to establish a sample sharing system across different anti-doping labs in the world using dried urine spots, which would be more cost-effective and efficient than urine liquid samples for discovery of emerging threats.

Future outlook for biomarkers in anti-doping control

Biomarker approaches have the potential to largely simplify the screening method, and continued innovation in this field is making it more difficult for people to cheat. We can already see some breakthroughs such as detection of GH using IGF-1 and P-III-NP

and the practice of ABP. There is little doubt that the use of biomarkers will play a pivotal and even irreplaceable role in anti-doping test in the future. Research into the use of metabolomics is still in its infancy but has great potential to significantly expand, and more and more studies are using metabolomics as a tool to identify new biomarkers for doping.